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# The Bromus tectorum-Pyrenophora semeniperda Pathosystem

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The *Bromus tectorum*-*Pyrenophora semeniperda* Pathosystem

Heather Finch

A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of

Master of Science

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June 2013

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## ABSTRACT

### The *Bromus tectorum*-*Pyrenophora semeniperda* Pathosystem

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Master of Science

Variable mortality of *Pyrenophora semeniperda*-infected *Bromus tectorum* seeds has been referred to as a “race for survival”, stating that seeds that germinate quickly are more likely to escape pathogen-caused mortality. Dormancy status is not the only variable determining outcomes within the *Bromus*-*Pyrenophora* pathosystem. Varying temperature and exposure to water may strongly influence germination outcomes of *B. tectorum* when in the presence of *P. semeniperda*. Low water potentials characteristic of semi-arid soils are often over-looked in the context of seed pathogens, and are ecologically relevant- especially for plant species that inhabit intermittently dry environments.

To adequately characterize the *Bromus tectorum*-*Pyrenophora semeniperda* pathosystem, four studies were conducted to address the following questions: (1) do temperature, water potential, and dormancy status influence germination outcomes in the *Bromus*-*Pyrenophora* pathosystem, (2) do repeated wetting-drying scenarios influence germination outcomes of infected *B. tectorum* seeds following dehydration at low water potentials similar to those found in the field (i.e., -4 through -150 MPa), (3) can we accurately characterize the asexual life cycle of *P. semeniperda* on a dormant *B. tectorum* seed, determining when infection takes place, and what occurs during disease development in continuously hydrated conditions, and (4) how does disease development of *P. semeniperda* influence the *B. tectorum* seed embryo and endosperm.

All studies were conducted using dormant and/or non-dormant *B. tectorum* seeds and an intermediate strain of *P. semeniperda*. Study one used varying temperatures (5-20°C), and five water potentials (0, -0.5, -1, -1.5, -2 MPa) (achieved using PEG 8000). Inoculated seeds were exposed to low water potentials at various temperatures for 7, 14, 21, or 28 days then re-hydrated for 28 days. In the second study, seeds were incubated at 20°C at four nominal water potentials (-4, -10, -40, or -150 MPa) following 8 or 24 hours of initial hydration. Seeds were dehydrated for 1, 7, 14, or 21 days, then re-hydrated. In study three, inoculated seeds were chemically fixed between days 0 and 21 and viewed with a scanning electron microscope. In the fourth study, infected seeds were frozen with liquid nitrogen following 3, 8, and 14 days of disease development, then cross sectioned longitudinally and laterally prior to chemical fixation.

Results indicate that non-dormant seeds escape death by germinating rapidly under favorable conditions, that incubation at low water potentials greatly increases seed mortality, that -10 MPa is near the threshold for full pathogen activity, and at water potentials lower than -40 MPa, *P. semeniperda* may successfully survive severe dehydration if previous hydration resulting in infection has occurred. SEM images indicate that mycelia penetration occurs within 8-24 hours, and that mycelium may penetrate all opening in the seed (i.e., stomata, cracks). Development of *P. semeniperda* is shown to cause significant damage to the endosperm and embryo within 8 days. As starch is consumed, the endosperm collapses leaving a hollow middle. The embryo is more resilient, but gradually deforms and deteriorates.

Keywords: *Bromus tectorum*, disease development, dormant, embryo, endosperm, germination, intermittent, mortality, non-dormant, pathosystem, *Pyrenophora semeniperda*, water potential, SEM

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## Chapter 1

Environmental factors influencing

*Pyrenophora semeniperda*-caused seed mortality in *Bromus tectorum*

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## ABSTRACT – ARTICLE 1

Temperature and water potential strongly influence seed dormancy status and germination of *Bromus tectorum*. As seeds of this plant can be killed by the ascomycete fungus *Pyrenophora semeniperda*, this study was conducted to learn how water potential and temperature influence mortality levels in this pathosystem. Separate experiments were conducted to determine: 1) if *P. semeniperda* can kill dormant or non-dormant seeds across a range of water potentials (0 to -2 MPa) at constant temperature (20°C), and 2) how temperature (5-20°C) and duration at reduced water potentials (0-28 days) affect the outcome. When inoculated with the fungus at 20°C, all dormant seeds were killed, but fungal stromata appeared more quickly at higher water potentials. For non-dormant seeds, decreasing water potentials led to reduced germination and greater seed mortality. Results were similar at 10 and 15°C. Incubation at 5°C prevented stromatal development on both non-dormant and dormant seeds regardless of water potential, but when seeds were transferred to 20°C, dormant seeds evidenced high mortality. For non-dormant seeds, exposure to low water potential at 5°C resulted in secondary dormancy and increased seed mortality. Increasing incubation temperature, decreasing water potential, and increasing duration at negative water potentials all led to increased mortality for non-dormant seeds. Results are consistent with field observations that pathogen-caused mortality is greatest when dormant seeds imbibe, or when non-dormant seeds experience prolonged or repeated exposure to low water potentials. We propose a conceptual model to explain the annual cycle of interaction in the *Bromus tectorum* – *Pyrenophora semeniperda* pathosystem.

Keywords: *Bromus tectorum*, pathosystem, *Pyrenophora semeniperda*, seed germination, temperature, time, water potential.

## INTRODUCTION

Seeds of the invasive annual grass *Bromus tectorum* L. exhibit dormancy at maturity and become increasingly germinable through dry after-ripening (Bair *et al.*, 2006). Hydrothermal time models have been developed to predict dormancy loss and germination under both laboratory and field conditions (Christensen *et al.*, 1996; Bauer *et al.*, 1998; Meyer and Allen, 2009). These models explain how the parameters time, temperature, and water potential influence the range of potential germination outcomes.

The ascomycete fungus *Pyrenophora semeniperda* has been shown to cause high mortality in dormant *B. tectorum* seed banks, and can kill non-dormant seeds as well (Meyer, *et al.*, 2007). Variable mortality of infected seeds is explained by Beckstead and others (2007) as a “race for survival”. This concept states that seeds that germinate quickly (e.g. fully after-ripened seeds incubated in water at optimum temperature) will be more likely to escape pathogen-caused mortality than seeds that germinate more slowly (Beckstead *et al.*, 2007).

Dormancy status is unlikely to be the only variable determining outcomes within the *Bromus tectorum*-*Pyrenophora semeniperda* pathosystem. Varying temperatures and water potentials, which can dramatically alter germination behavior, may likewise alter the fate of seeds exposed to this pathogen. For example, if seeds imbibe but do not remain sufficiently hydrated for radicle emergence to occur, *P. semeniperda* might still be able to infect and kill seeds. Studies on the combined effect of temperature and water availability to fungi including *Penicillium expansum*, *Penicillium citreoviride*, *Penicillium citrinum*, *Fusarium moniliforme*, and *Fusarium proliferatum* have shown that these pathogens can grow and thrive at negative water potentials (Lahlali *et al.*, 2005; Ji *et al.*, 2007; Marin, *et al.*, 1996). In fact, optimum

growth occurred at various water potentials ranging from -2.5 MPa to -14.5 MPa (Lahlali *et al.*, 2005; Ji *et al.*, 2007; Marin, *et al.*, 1996).

The present study was conducted to learn how temperature and water potential influence the *Bromus-Pyrenophora* pathosystem. We specifically sought to determine if *P. semeniperda* can infect and kill dormant and non-dormant seeds across a range of water potentials at constant temperature, and how the variables temperature and duration at reduced water potentials affect the fate of seeds. Results will allow us to better understand the seed-pathogen interaction, and develop a conceptual framework to explain potential outcomes under a wide range of conditions including those likely to occur in the field.

## MATERIALS AND METHODS

Seeds of *Bromus tectorum* L. were collected from a wild population at the Brigham Young University Research Farm (Spanish Fork, Utah, USA) in June 2009. Seeds were cleaned by hand and stored in one of two ways: under ambient laboratory conditions to allow seeds to after-ripen, or in a -10°C freezer to maintain seeds in the dormant condition. 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). Seeds in all experiments were inoculated with a 1:100 spore:talc mixture by placing seeds and an excess of inoculum in a test tube vial and shaking for 30 seconds. The first two experiments were conducted in 2010. The third experiment was conducted in 2012.

In the first experiment, inoculated dormant or fully after-ripened (non-dormant) seeds (50 seeds X 4 replicates) were imbibed at constant 20°C, which is near optimum for the pathogen (Campbell *et al.*, 1995) and is also a typical mean autumn temperature during germination

triggering rainfall events (Meyer and Allen, 2009). Seeds were exposed to cycles of 12 hour fluorescent light/12 hour dark at one of five nominal water potentials (0 MPa, -0.5 MPa, -1 MPa, -1.5 MPa, -2 MPa) achieved using solutions of Polyethylene glycol 8000 as described by Michele and Kaufmann (1972). Seeds were placed in Petri dishes on the surface of two blue germination blotters (Anchor Paper, St. Paul, Minnesota, USA) that had been saturated to excess with the appropriate solution. Dishes were placed in plastic sleeves and then tilted at an angle of approximately 20 degrees to allow a pool of polyethylene glycol to remain at the bottom of the dishes, preventing blotters from drying out and minimizing changes in water potential. Dishes were incubated for 28 days (hereafter referred to as a “pretreatment”). Germinated (radicle protruded at least 1 mm) or killed (macroscopic *P. semeniperda* stromata visible with no radicle present, Figure 1) seeds were counted and removed on days 2, 4, 7, 11, 14, 21, and 28. On day 28, all remaining seeds were transferred from pretreatment to new Petri dishes containing two blotters saturated with water (0 MPa) and incubated for an additional 28 days. Germinated and/or killed seeds were again counted on days 2, 4, 7, 11, 14, 21, and 28. Remaining seeds were scored as viable but dormant if firm when pressed.

The second experiment was a factorial design that included two dormancy states (dormant or fully after-ripened) X four constant incubation temperatures (5, 10, 15, 20°C in 12:12 hour light:dark cycles) X three incubation water potentials (0 MPa, -1.5 MPa, -2 MPa) X four pretreatment periods (7, 14, 21 or 28 days) prior to transfer to water (0 MPa at the same incubation temperature as for initial incubation) for 28 days X two replicates (50 seeds/replicate). The temperature range for this experiment (5-20°C) included germination-permissive temperatures that can be experienced by seeds during and/or after the first germination triggering rainfall event (Meyer and Allen, 2009). Seeds were incubated and scored for germination or

death as in the first experiment, except that scoring at low water potentials on days 11, 14, 21, and 28 days was possible only for treatment durations that included these days.

Based on results of the second experiment, a small third experiment was conducted using seeds collected in 2011 from the same site as the earlier experiments. Dormant or after-ripened seeds (as previously described) were pretreated at 5°C (0, -1.5, or -2 MPa) for 14, 21, or 28 days, then transferred to water (0MPa) at 20°C for 28 days. Seeds (4 replicates of 25 seeds) were scored for germination or death as previously described.

Experimental data were analyzed as fully randomized designs using the analysis of variance (ANOVA) procedure of SAS (SAS 9.2, 2007). Data were arcsine transformed for analysis to account for heterogeneity of variance. However, original means are reported. Means separations were performed as appropriate using Duncan's multiple range test. In the second experiment, the treatment in which seeds were placed directly in water with no pretreatment was included in the analysis as a zero-duration pretreatment.

## **RESULTS**

In the first experiment, nearly all dormant seeds were killed by the fungus, either during incubation (20°C) at low water potentials (-0.5, -1 MPa pretreatments) or following transfer to water (-1.5, -2 MPa pretreatments) (Figure 2A). When dormant seeds were incubated directly in water, fungal stromata indicating seed death (Figure 1) appeared between 14 and 21 days (Figure 2A, "no pretreatment" seeds). For dormant seeds incubated at -0.5 or -1 MPa, stromata most commonly appeared during the 28-day pretreatment period, while dormant seeds incubated at -1.5 or -2.0 MPa exhibited stromatal development within four days following transfer to water (Figure 2A). In contrast, few non-dormant seeds were killed during pretreatment at higher water

potentials. Following transfer to water, less than 20% of seeds previously incubated at -0.5 or -1 MPa were killed (Figure 2B). However, pretreatment at the lowest water potentials resulted in non-dormant seed death of 63% (-1.5 MPa) or 75% (-2 MPa) following transfer to water. The appearance of *P. semeniperda* stromata on ungerminated seeds was used to define seed death. However, based on the rapid appearance of stromata following transfer to water, seeds were certainly infected and may actually have been killed during pretreatment. Incubation at the lowest water potentials apparently prevents the development of stromata, analogous to seed priming treatments wherein incubation at low water potentials can allow progress toward germination while restricting radicle emergence (Taylor *et al.*, 1998). Nearly all non-dormant seeds incubated at 0 or -0.5 MPa germinated (Figure 2C). Fewer seeds germinated as pretreatment water potential decreased, even following transfer to water, because they had been killed.

In the second experiment, incubation at the higher temperatures resulted in dramatic mortality of dormant seeds (Figure 3). One-hundred percent of dormant seeds were killed at 15 and 20°C (Figure 3A, B, E, F), regardless of incubation water potential or duration of the low water potential pretreatment. Mortality at 10°C ranged from 60% (incubation in water only) to nearly 100% (incubation at -2 or -1.5 MPa for 28 days) (Figure 3 C, G). Differences between water potentials (-2 versus -1.5 MPa) were not significant, while water potential duration was marginally significant ( $P=0.04$ ), probably due to increased mortality with longer incubation at 10°C.

Incubation at 5°C resulted in pathogen-caused death to less than 10% of dormant seeds when incubated only in water (0 MPa, Figure 3 D, H). Over 60% of putatively dormant seeds germinated in water, indicating that they were only conditionally dormant as a function of



temperature. Incubation at -2 or -1.5 MPa rendered these conditionally dormant seeds incapable of germinating even after transfer to water for 28 days.

For non-dormant seeds, all main effects (temperature, water potential, duration) had highly significant impacts on levels of seed mortality ( $P < 0.0001$ ). At 15 and especially at 20°C, a large fraction of non-dormant seeds were killed by the fungus with low water potential incubation periods of 14 days or greater (Figure 4 A, B, E, F). Almost complete mortality of non-dormant seeds occurred after incubation at -2 MPa for 21 or 28 days. Prolonged incubation at low water potentials (>14 days) resulted in a progressive increase in the fraction of non-dormant seeds killed. At 10 or 15°C, -1.5 MPa, incubation resulted in considerable germination prior to transfer to water (Figure 4 F, G). At 10°C incubation at low water potentials for 0 or 7 days, most non-dormant seeds germinated following transfer to water (Figure 4 C, G). Less than 5% of non-dormant seeds were killed during incubation at 5°C (Figure 4 D, H). All seeds germinated in water (i.e., no subzero water potential pretreatment) at this temperature, while increasing duration at low water potentials rendered a progressively greater fraction of the seeds dormant (i.e., secondarily dormant).

These results at 5°C prompted an additional experiment to evaluate whether non-dormant seeds at low incubation temperature were infected and killed at low water potentials, but with evidence of seed death (stromatal growth) inhibited at low temperature. If this were the case, we would expect rapid growth of stromata following transfer to water at 20°C. Few seeds incubated in water at 5° were killed (Figure 5 A-F). However, some developed stromata following transfer to 20°C (<20% of non-dormant and < 30% of dormant seeds). This suggests that the fungus can infect at 5°C in water, but because even conditionally dormant seeds can germinate under these

conditions, the fungus is not highly effective at killing seeds at low incubation temperatures in water.

Seeds transferred from low water potentials at 5°C to water at 20°C were largely killed over time, but when rate of stomatal development is compared to the rate at which previously unimbibed seeds developed stomata (“0 MPa, no pretreatment” seeds in Figure 2A), it is possible that seeds that developed stomata later than about 14 days following transfer to water at 20°C were infected after transfer. Conversely, it is possible that incubation at low temperature and low water potential affects the rate at which the fungus can produce stomata when transferred to higher temperature. Using “visible stomata on ungerminated seeds” as our indicator of seed death did not allow us to clearly distinguish seeds that were infected during the low temperature incubation from those that may have been infected later.

## DISCUSSION

Results from the present study confirm that when *Bromus tectorum* seeds are incubated in the presence of *Pyrenophora semeniperda*: 1) dormant seeds are likely to be killed under all conditions except non-limiting water at low temperature, 2) non-dormant seeds escape death by germinating rapidly under favorable conditions, 3) incubation at low water potentials restricts germination of non-dormant seeds and greatly increases seed mortality, 4) non-dormant seeds held at low water potentials for increasing periods of time are more likely to be killed, and 5) incubation at low temperature and low water potential induces secondary dormancy, leading to death if seeds are transferred to water at a higher temperature. These insights into the *Bromus tectorum*-*Pyrenophora semeniperda* pathosystem can be combined with results from field studies to create a conceptual model that explains the influences of time, temperature and water potential

throughout the year (Figure 6). This model expands the “race for survival” concept originally proposed by Beckstead *et al.*, (2007).

In the absence of the fungus, *B. tectorum* seeds exhibit behavior characteristic of winter annual grasses (Figure 6, inner circle). Seed populations have varying degrees of dormancy at maturity, and gradually lose dormancy through dry after-ripening. After-ripened seeds germinate in response to autumn rains, postpone germination until winter or early spring, or acquire secondary dormancy and carry seeds across years as components of the soil seed bank (Allen and Meyer 2002).

As indicated by levels of *Pyrenophora*-killed seeds retrieved from soil seed banks, seeds mature at a time when maximum levels of the fungus are present (Beckstead *et al.*, 2007; Meyer *et al.*, 2007); precipitation at this stage could potentially result in a high degree of infection (Figure 6, outer circle 1). Surviving carryover seeds, which became secondarily dormant during the previous winter, are likewise present in the soil seed bank and experience similar vulnerability to the fungus. Most seeds are incapable of germinating at soil temperatures likely to be encountered during the summer, and precipitation is unlikely to wet the soil long enough for radicle emergence to be completed for the fraction of the seed population that is capable of germinating (Meyer and Allen, 2009). In the presence of *P. semeniperda*, seeds infected during the summer are likely to be killed.

As seeds lose dormancy through dry after-ripening during summer and early autumn (Bair *et al.*, 2006), they have the potential to experience repeated imbibition episodes followed by drying. Rapid growth of stromata following transfer of seeds from low water potentials to water suggests that the fungus can likely grow and infect seeds over a series of hydration-dehydration events (Figure 6, outer circle 2). However, an alternative outcome is possible for

non-dormant seeds that become infected. With adequate moisture, seeds germinate quickly and avoid being killed by the fungus. This describes the only opportunity for successful seed germination following infection (Figure 6, outer circle 2), and is supported by field studies showing that moderate temperatures associated with autumn storms kept the soil surface from drying (Meyer and Allen, 2009).

During late summer and early autumn, uninfected seeds will germinate in response to an autumn germination triggering rainfall event, while seeds already infected during previous storms are likely to be killed before they can germinate. After-ripening of *Bromus tectorum* is associated with an increase in germination rate as well as an increase in the temperature range that allows germination (Christensen *et al.*, 1996). Seeds that germinate quickly are more likely to escape seed death than slow-germinating seeds. In the field, successful germination is most likely to occur during autumn, especially when fully after-ripened seeds encounter their first imbibition experience associated with a germination triggering rainfall event at optimum temperatures.

In a field study aimed at characterizing the relationship between *B. tectorum* and *P. semeniperda*, wet autumn weather at one site allowed non-dormant seeds to germinate quickly, preventing secondary dormancy induction and associated pathogen-caused mortality (Beckstead *et al.*, 2007). In this same study, sites receiving low levels of autumn precipitation had large numbers of seeds that became secondarily dormant. These dormant seeds carried over during the winter in the soil seed bank, with subsequently high pathogen-caused mortality. As a result, these drier sites often had high levels of pathogen-killed secondarily dormant seeds retrieved from soil seed banks in the spring. This interaction sequence is depicted in Figure 6 as outer circle 3.

Precipitation at any time during the year likely permits *P. semeniperda* to infect seeds, including late autumn or winter (Figure 6). Fully hydrated *B. tectorum* seeds can complete germination slowly at low temperatures during the winter, although low water potentials at near-freezing temperatures can induce secondary dormancy. Secondarily dormant *B. tectorum* seeds are highly susceptible to *P. semeniperda* under laboratory (Figure 5) and field (Beckstead *et al.*, 2007; Meyer *et al.*, 2007) conditions. In studies of soil seed banks conducted throughout the year on sites where the *Bromus-Pyrenophora* pathosystem is known to occur, we have repeatedly observed the highest numbers of fungus-killed seeds in late spring (Figure 6, outer circle sequence 3; S. Meyer, unpublished data). Results from the present study suggest that dormant seeds may be killed during the winter but the evidence of death (presence of stromata) only appears at higher temperatures.

*P. semeniperda* can clearly infect seeds at negative water potentials as well as in water (0 MPa), although the outcome (seed death versus germination success) may depend on the sequence of environmental variables encountered (e.g., repeated hydration followed by dehydration, temperature fluctuations). Seed dormancy status, water potential, and the interaction of these variables with temperature all contribute to the duality of outcomes (seed germination or seed death). Certain conditions clearly favor the fungus (highly dormant seed population, prolonged exposure to low water potential, non-optimal germination temperature) while other conditions (after-ripened seed population, optimum hydrothermal environment) favor successful germination. Based on our understanding of hydrothermal time and germination of *B. tectorum* seeds (Bair *et al.*, 2006; Bauer *et al.*, 1998; Christensen *et al.*, 1996; Meyer and Allen 2009), it is likely that the fastest-germinating seed fractions (i.e., those with the lowest base water potentials) are also those most likely to likely to escape death due to the fungus. High germination rates for

after-ripened seeds, which are associated with low population mean base water potentials in hydrothermal models (Bair *et al.*, 2006; Meyer and Allen, 2009), may in part be the result of selection pressure imposed by this pathogen.

It has long been known that certain plant pathogens can infect and kill seeds at water potentials far below those that permit seed germination (e.g., Magan and Lacey, 1988). However, to our knowledge this study represents the first investigation of the consequences of seed infection at low water potential in a natural pathosystem. The ecological implications of this work are profound, especially for plant species that inhabit intermittently dry environments. Seeds of such species are likely to spend extended periods of time at water potentials conducive to pathogen attack. Plant pathogens are rarely considered in studies of desert ecosystems but these pathogens, particularly those that attack seeds, could potentially function as keystone organisms with major impacts on desert plant community structure (Dobson and Crawley, 1994).

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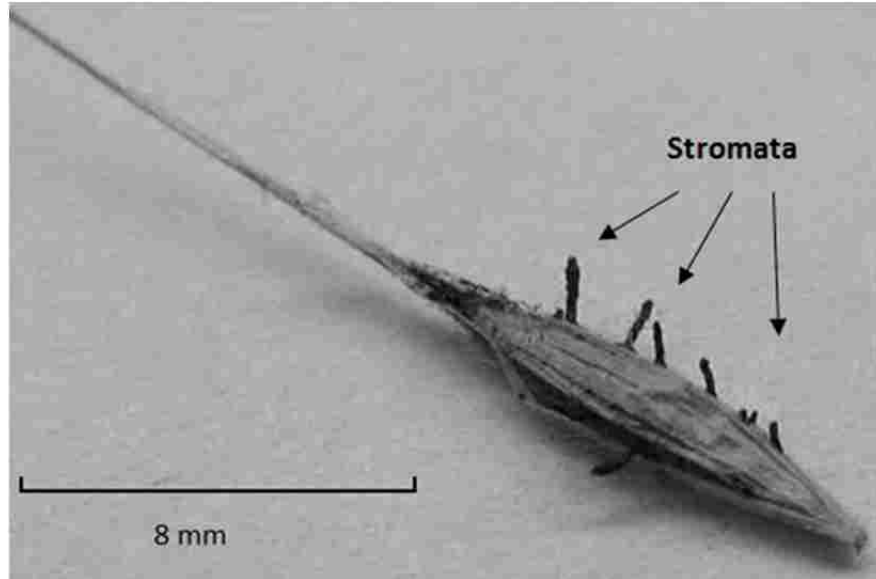
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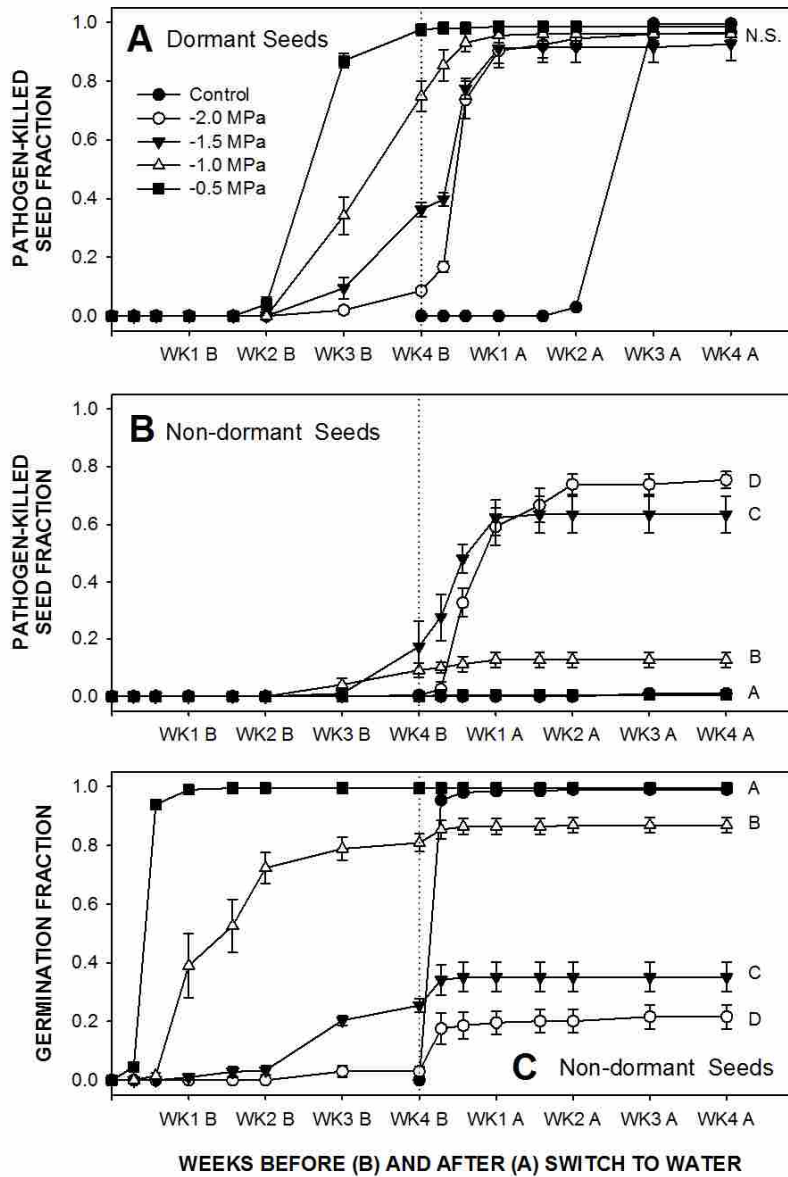


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## FIGURES

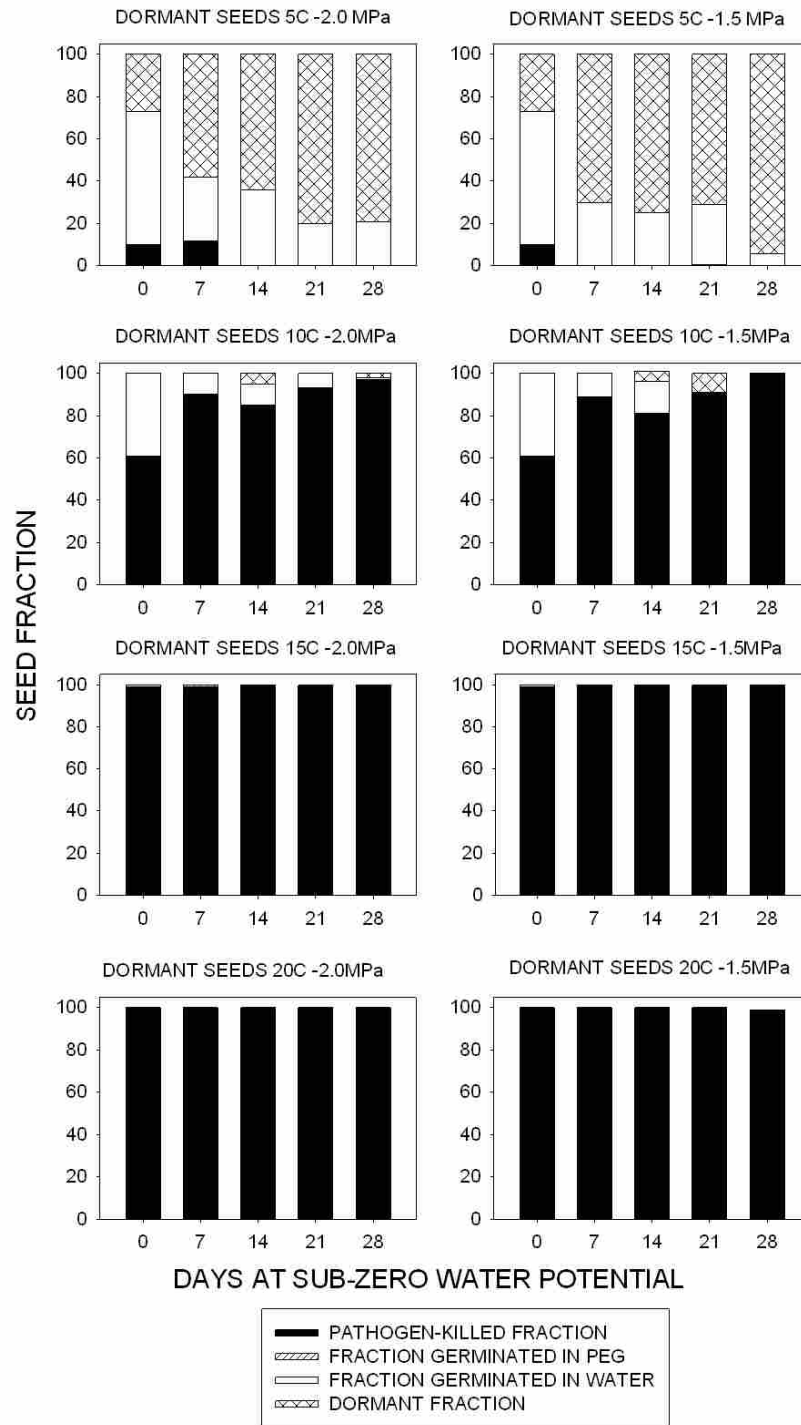


**Figure 1. Microscopic View of a *Bromus tectorum* Seed Killed by *Pyrenophora semeniperda*.** Emergence from an ungerminated seed of one to several finger-like stromata, fruiting bodies that produce spores, is evidence of seed death.

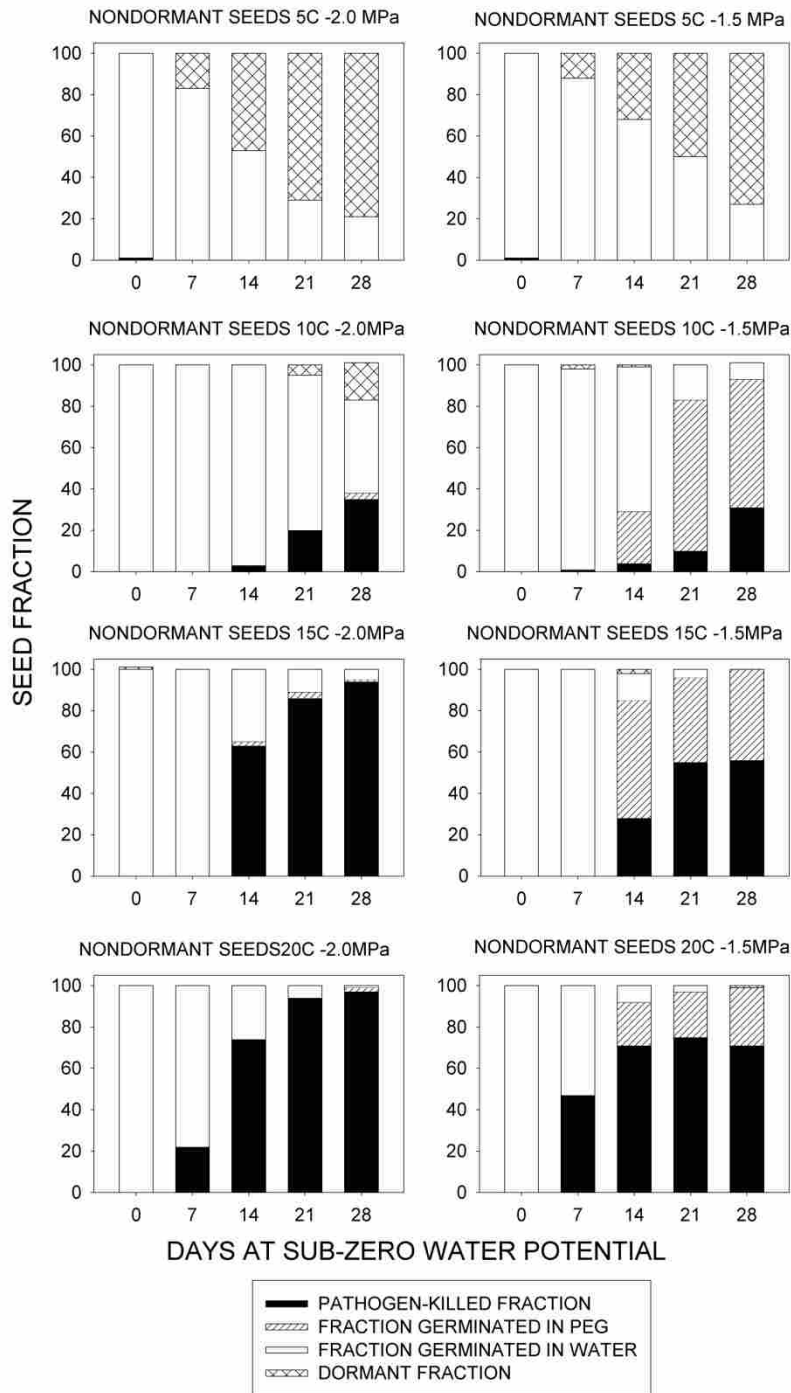


**Figure 2. Germinated or Killed *B. tectorum* Seeds Before and After Switch to Water.**

A) Mortality of dormant *Bromus tectorum* seeds when incubated in the presence of *Pyrenophora semeniperda*, B) mortality of non-dormant seeds, and C) germination of non-dormant seeds, each as a function of time in incubation at 20<sup>o</sup> C. Seeds were exposed to five water potentials for four weeks followed by incubation in water (0 MPa) for an additional four weeks. Vertical dotted lines mark transfer to water. Error bars represent the standard error of the mean. Final values associated with different letters (e-h) are significantly different (P<0.05) as determined by a Duncan multiple range means separation test following ANOVA.

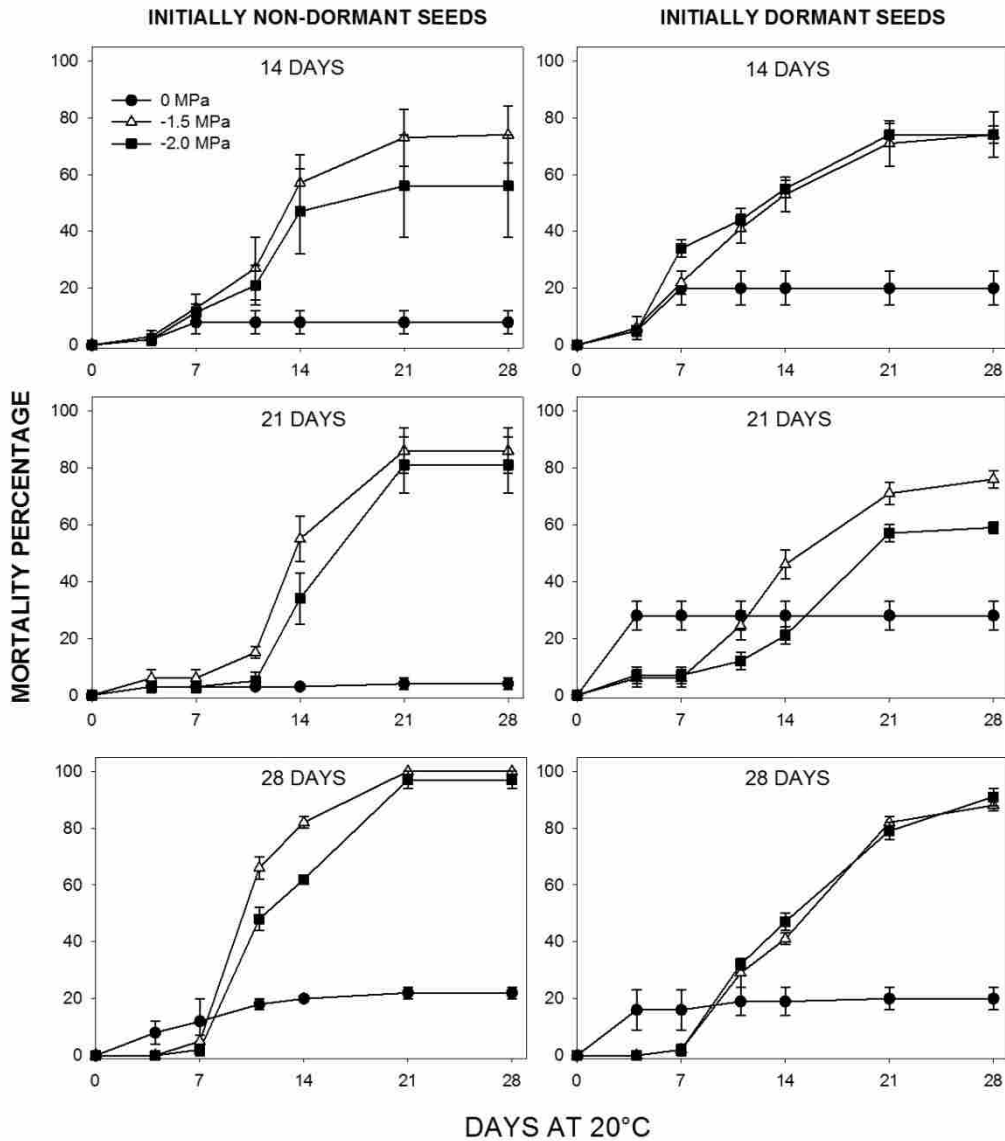


**Figure 3. Percentage of Initially Dormant Germinated and *P. semeniperda*-Killed *B. tectorum* Seeds.** Percentage of initially dormant *B. tectorum* seeds killed by *P. semeniperda*, germinated after transfer to water, or dormant (viable ungerminated) after 0 to 28 days pretreatment followed by 28 days in water. Seeds with no pretreatment (0 days at sub-zero water potentials) were only incubated in water (0 MPa). Pretreatments included a factorial combination of two sub-zero water potentials (-2 MPa, A-D or -1.5 MPa, E-H) and four incubation temperatures: 20°C (A, E); 15°C (B, F); 10°C (C, G); and 5°C (D, H). Seeds remained at pretreatment temperatures when transferred to water.



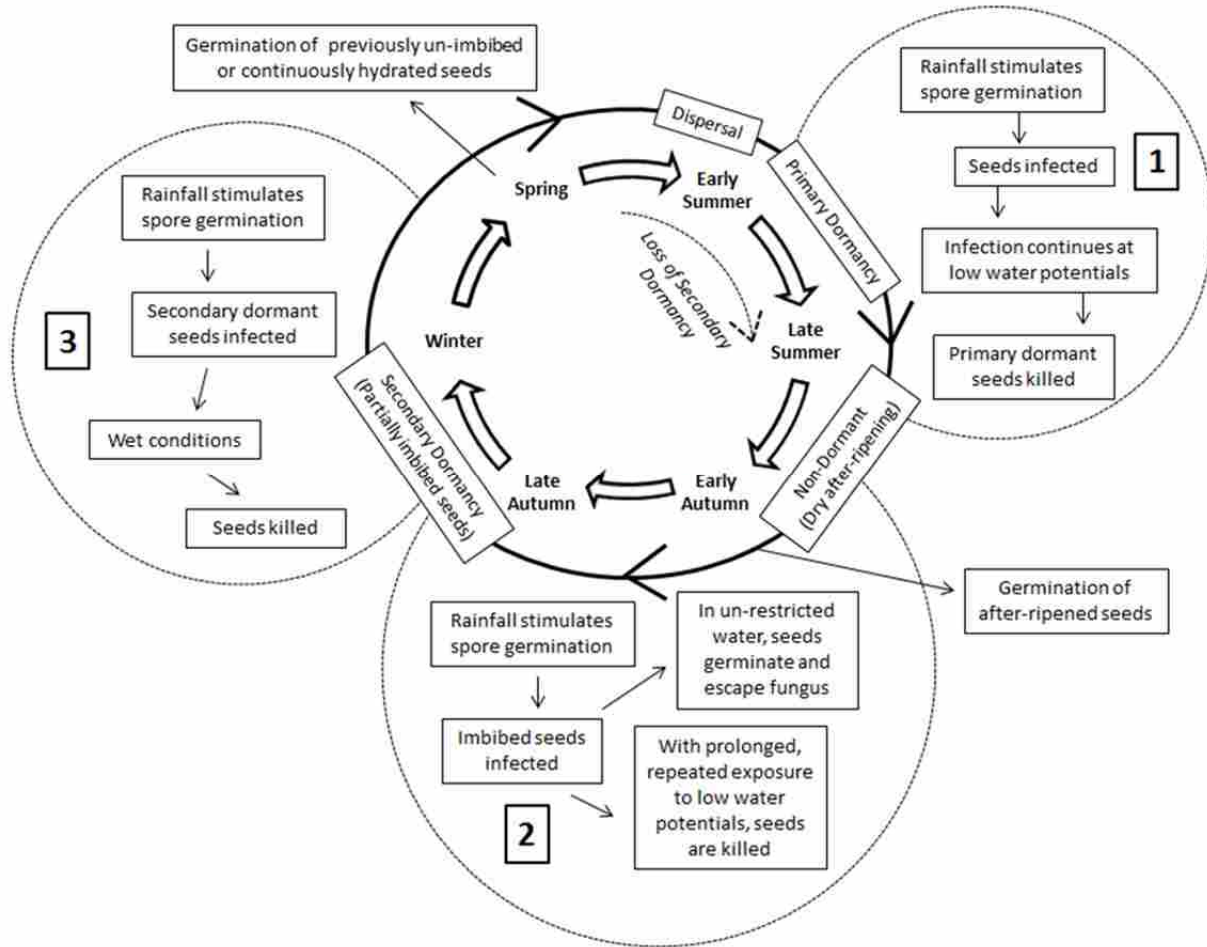
**Figure 4. Percentage of Initially Non-dormant Germinated and *P. semeniperda*-Killed *B. tectorum* Seeds.**

Percentage of initially non-dormant *B. tectorum* seeds killed by *P. semeniperda*, germinated in pretreatment, germinated after transfer to water, or dormant (viable ungerminated) after 0 to 28 days pretreatment followed by 28 days in water. Seeds with no pretreatment (0 days at sub-zero water potentials) were only incubated in water (0 MPa). Pretreatments included a factorial combination of two sub-zero water potentials (-2 MPa, A-D or -1.5 MPa, E-H) and four incubation temperatures: 20°C (A, E); 15°C (B, F); 10°C (C, G); and 5°C (D, H). Seeds remained at pretreatment temperatures when transferred to water.



**Figure 5. Mortality of *B. tectorum* Seeds Inoculated with *P. semeniperda*.**

Seeds were pretreated (5°C) at water potentials indicated for 14 (A, D), 21 (B, E) or 28 (C, F) days, then transferred to water (20°C). No mortality as indicated by stomatal growth occurred during pretreatment. Error bars represent the standard error of the mean.



**Figure 6. The *Bromus tectorum*-*Pyrenophora semeniperda* Pathosystem in a Semi-arid Environment.**

In the absence of the fungus, *B. tectorum* seeds follow the typical cycle for a winter annual (inner circle). Numbered outer circles indicate three main periods with different fungal-seed interaction sequences. 1) Summer: infected seeds, across a range of water potentials, are killed by the fungus; 2) Autumn: infected seeds escape death with sufficient rainfall due to rapid germination or are killed if radicle emergence is delayed by exposure to low water potentials; 3) Winter/Spring: fully imbibed secondarily dormant seeds are killed.

## Chapter 2

Effect of intermittent hydration following exposure to the pathogen

*Pyrenophora semeniperda* on *Bromus tectorum* seed mortality

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## ABSTRACT- ARTICLE 2

Water availability and dormancy status influence seed outcomes in the *Bromus tectorum*-*Pyrenophora semeniperda* pathosystem. Because seeds are often exposed to intermittent hydration, pathogenicity of *P. semeniperda* may be favored if disease development is permitted at germination-inhibiting water potentials. To study mortality following exposure to widely fluctuating water potentials, inoculated *B. tectorum* seeds were hydrated until they a) were just imbibed, or b) had nearly completed germination, and then subjected to varying degrees of dehydration at low water potentials ( -4 to -150 MPa) for up to 21 days followed by rehydration. Intermittent hydration greatly increased mortality of non-dormant seeds, but the magnitude was dependent upon the interaction between initial hydration (8 vs. 24 hours), dehydration water potential and dehydration duration (1 to 21 days). Mortality of initially dormant seeds was reduced by 25% due to dormancy-breaking associated with intermittent hydration and low water potentials. Our data show that -10 MPa is near the threshold for full pathogen activity (i.e., seed mortality progressively decreases at lower water potentials) and that approximately 70% of non-dormant seeds were killed by *P. semeniperda* when exposed to intermittent hydration. Successful infection prior to dehydration permitted some pathogen activity at dehydration water potentials as low as -40 MPa. While pathogen activity did not occur at -150 MPa, the fungus still survived within seeds and could kill seeds upon rehydration. To our knowledge, this is the first time a seed-fungal pathosystem has been explored in the context of widely fluctuating water potentials.

Keywords: *Bromus tectorum*, germination, intermittent hydration, mortality, *Pyrenophora semeniperda*, water potential.

## INTRODUCTION

The fate of seeds in natural ecosystems, especially those involved in host-parasite interactions, is dependent on the sequence of environmental conditions that occur from seed development through germination and seedling establishment. From the perspective of a mature seed population, water availability is critically important. For the invasive winter annual grass *Bromus tectorum*, summer dormancy loss through after-ripening (Bair *et al.*, 2006), autumn germination (Bauer *et al.*, 1998; Meyer and Allen, 2009), and secondary dormancy induction (Finch *et al.*, 2013; Allen *et al.*, 2010) are all influenced by the sequence of water potentials to which seeds are exposed.

Fungi that attack seeds are also influenced by water availability. Not surprisingly, the majority of published studies regarding how seed pathogens are influenced by water potential have been conducted on fungi that attack stored grains (Torres *et al.*, 2002; Ramos *et al.*, 1998; Lacey and Magan, 1991). In the context of *in situ* soil seed banks, few reports on seed pathogens have been published even though seed-fungi interactions can have major ecological significance in wildland systems (Gilbert, 2002; Chambers and MacMahon, 1994). The *Bromus tectorum* - *Pyrenophora semeniperda* pathosystem is one of the few for which published studies exist (Meyer *et al.*, 2007; Finch *et al.*, 2013). The ascomycete fungus *Pyrenophora semeniperda* can cause high mortality to dormant *B. tectorum* seeds in the soil, but can kill non-dormant seeds as well (Meyer *et al.*, 2007; Finch *et al.*, 2013).

*Bromus tectorum* seeds dehisce from the plant as at least conditionally dormant populations, and lose dormancy through dry after-ripening (Christensen *et al.*, 1996; Allen, 2003). While autumn precipitation may trigger germination, rate and percentage are controlled by dormancy status, fluctuations in soil water potential, and temperature (Meyer and Allen,

2009). Non-dormant seeds generally escape *P. semeniperda* through rapid germination (Beckstead *et al.*, 2007), although low water potentials (-1 to -2 MPa) were shown to inhibit seed germination and favor the pathogen (Finch *et al.*, 2013). Seeds under field conditions can experience occasional or frequent exposure to far lower water potentials following initial imbibition. We therefore decided to study germination outcomes of inoculated *B. tectorum* seeds when subjected to widely fluctuating water potentials typically encountered in semi-arid soils.

The objectives of this study were to first, determine if exposure of seeds imbibed in the presence of *P. semeniperda* then dried at water potentials from -4 to -150 MPa would permit infection and eventual mortality and second, how *P. semeniperda*-caused mortality is influenced by duration of imbibition prior to dehydration at low water potentials. We hypothesized that longer initial hydration prior to dehydration at low water potentials would benefit the fungus, and that disease development of *P. semeniperda* was limited to higher water potentials (> -4 MPa). We addressed these hypotheses by hydrating inoculated seeds until the rapid phase of imbibition was complete (i.e., near the end of phase one of water uptake, Bewley *et al.*, 2013) or until seeds had nearly completed germination (i.e., near the end of the lag phase of water uptake), and then subjecting them to varying degrees of dehydration at low water potentials before rehydrating them.

## METHODS

Seeds of *B. tectorum* L. were collected from a wild population at the Brigham Young University Research Farm (Spanish Fork, Utah, USA) in June 2011. Seeds were cleaned by hand and stored in one of two ways: under ambient laboratory conditions to allow seeds to after-ripen, or in a -10°C freezer to maintain primary seed dormancy. The *P. semeniperda* inoculum

originated as a moderately virulent strain collected from Whiterocks, Utah, USA, and was produced as described by Meyer *et al.*, (2010). Seeds in all experiments were inoculated with a 1:100 spore:talc mixture (w/w) by placing seeds and an excess of inoculum in a test tube vial and rapidly shaking for 30 seconds. Inoculated seeds were then exposed to initial hydration (i.e., imbibition) followed by controlled dehydration then rehydration. Hydration and dehydration treatments had regular and predictable effects on seed water content (Figure 1).

Non-dormant (fully after-ripened) and dormant seeds (25 seeds X 4 replicates) were imbibed in Petri dishes on the surface of two blue germination blotters (Anchor Paper, St. Paul, Minnesota, USA) at constant 20°C (12 h fluorescent light/12 h dark cycle), which is near optimum for the pathogen (Campbell *et al.*, 1995) and is also a typical mean autumn temperature during germination-triggering rainfall events (Meyer and Allen, 2009). Seeds were hydrated continuously or transferred to controlled drying environments following initial hydration periods of either 8 (i.e. “Early Dehydration”, Figure 1) or 24 hours (i.e., “Late Dehydration”) (20°C, dark conditions). Dehydration water potentials included -4, -10, -40, and -150 MPa, achieved above saturated salt solutions (K<sub>2</sub>SO<sub>4</sub>, KNO<sub>3</sub>, NaCl and MgCl<sub>2</sub>, respectively) in sealed containers. Seeds were placed on plastic weighing trays and floated above salts in sealed jars for durations of either 1, 7, 14, or 21 days (Allen *et al.*, 1992; Allen *et al.*, 1993a). Infected dormant seeds were incubated under similar conditions, except that dormant seeds were only dehydrated for 14 days.

Following dehydration, seeds were returned to water-saturated blotters and rehydrated. Seeds were then scored as germinated (radicle protruded at least 1 mm) or killed (macroscopic *P. semeniperda* stromata visible with no radicle present), on days 2, 4, 7, 11, 14, 21 and 28.

## RESULTS

Water uptake during initial hydration followed a characteristic triphasic pattern that included a rapid imbibition phase (0-8 hours) followed by a period of slower water uptake (8-32 hours) after which radicle emergence and an increased rate of water uptake in developing seedlings was observed (Fig. 1). Dehydration initiated after 8 or 24 hours resulted in rapid seed water loss. Seeds arrived at near-equilibrium water contents at each water potential; seeds initially hydrated for 8 hours reached near-equilibrium within six hours, while seeds initially hydrated for 24 hours reached near-equilibrium within 14 hours. Dehydration at the lower water potentials removed water more rapidly and resulted in lower seed water contents at equilibrium. Dehydration at -150 MPa returned seeds to near air dry weights within 24 hours.

In the control treatment (continuous hydration), dormant seeds experienced very high mortality and non-dormant seeds experienced very low mortality (Fig. 2) confirming results of a previous study, (Finch *et al.*, 2013). For dormant seeds, hydration-dehydration-rehydration treatments actually reduced mortality in some treatments; dehydration at low water potentials reduced dormancy, permitting a fraction of seeds to escape by germinating before the fungus killed the seeds. For non-dormant seeds, hydration-dehydration-rehydration treatments increased mortality. With dehydration at -4 MPa, mortality of dormant and non-dormant seeds was high and did not differ between short and long initial hydration treatments.

With dehydration at lower water potentials, seed mortality was dependent on initial hydration (short vs. long) and seed dormancy status (Fig. 2). Up to 25% of initially dormant seeds were able to escape mortality through germination during the rehydration period. More seeds escaped when initial hydration was short, likely due to lack of fungal penetration within

the seed caryopsis. This was most evident at the two lowest water potential treatments (-40 and -150 MPa).

Initially non-dormant seeds suffered higher mortality when longer initial hydration was followed by dehydration at low water potentials (Fig. 2). At -10 MPa, mortality with early dehydration was still relatively high, but at water potentials lower than -10 MPa, there was very little mortality. With late dehydration, mortality remained relatively high even when seeds were dehydrated at low water potentials. Dehydration at -150 MPa coupled with late dehydration resulted in nearly 50% mortality, probably because rapid dehydration to very low water potentials delayed and desynchronized the seeds (Debaene *et al.*, 1994), making the slowest germinating fraction more vulnerable to mortality during subsequent rehydration.

Disease development of *P. semeniperda* continued in seeds during many dehydration treatments, and resulted in higher mortality with longer dehydration periods (Fig. 3). At -4 MPa, mortality increased with time following dehydration durations of 1 and 14 days, then leveled off at >90% following dehydration for 21 days. This pattern was the same for seeds subjected to either short or long initial hydration periods. At -10 MPa, the pattern of increasing mortality with increasing dehydration duration was similar to that for -4 MPa, but not as pronounced. With 14 days of dehydration, seed mortality averaged about 50% with no further increase in mortality with dehydration for 21 days.

At -40 MPa, there was almost no mortality with early dehydration regardless of dehydration duration. Successful infection of seeds in this treatment did not occur before the rehydration period, and consequently allowed many seeds to escape the fungus through germination. Results following late dehydration appear to indicate that, once the seed is infected during the initial hydration period, the fungus can continue disease development within the seed

even at -40 MPa, as post-dehydration mortality showed a very clear linear increase with dehydration time. Mortality with 21 days of dehydration at -40 MPa was almost as high for seeds dehydrated at -4 MPa. It is a matter of speculation how the fungus maintained its effectiveness at this low water potential.

At -150 MPa, the fungus was unable to cause high mortality with early dehydration (Fig. 3). With late dehydration, mortality percentage was maintained at a relatively constant level regardless of dehydration duration, indicating that the pathogen was unable to operate inside the seed at this low water potential. The roughly 30% mortality observed may be partially due to the deleterious effect of rapid, late dehydration on seed vigor resulting in slowed germination accompanied by higher mortality, or the induction of secondary dormancy. This is consistent with the observed pattern of essentially constant mortality across all dehydration periods.

Time course curves for mortality of non-dormant seeds are shown in Figure 4. Early dehydration for 21 days resulted in rapid development of fungal stromata starting just 4 days following rehydration for seeds dehydrated at -4 MPa. However, stromatal development with early dehydration was only slightly accelerated when seeds were dehydrated at -10 MPa, and very little mortality occurred for seeds dehydrated at -40 or -150 MPa. Dehydration durations of 7 and 14 days showed an intermediate response (data not shown).

For seeds subjected to late dehydration, results for seeds dehydrated at -4 MPa were similar (Fig. 4). Seeds began developing stromata within 4 days of rehydration. At -10 MPa, fungal stromata developed by day 7, faster than with early dehydration. At -40 MPa, stromatal development was not much faster than for continuously hydrated seeds, (at -40 MPa, appearance occurred at day 11 of rehydration) even though mortality was high. At -150 MPa, stromatal

development was delayed until 11 days after rehydration, indicating that little disease development had occurred inside the seed during the dehydration treatment.

## DISCUSSION

Results from this study indicate that: (1) intermittent hydration can greatly increase mortality of non-dormant *B. tectorum* seeds, but the magnitude of the effect depends on the interaction between initial hydration period, dehydration water potential, and dehydration duration, (2) mortality of initially dormant *B. tectorum* seeds was actually reduced somewhat by intermittent hydration, apparently associated with a degree of dormancy-breaking effect when dormant, imbibed seeds were dehydrated at low water potentials, and (3) time in post-dehydration incubation required for the appearance of stomata was related to the imbibition period and the length and severity of the dehydration treatment; early dehydration at the lowest water potentials failed to advance disease development as compared to continuously hydrated seeds regardless of whether seeds were subjected to early or late dehydration duration. In contrast, with late dehydration at the highest water potential, the time requirement for stomata development was shortened to as little as four days.

Late dehydration resulted in a larger proportion of pathogen-killed non-dormant seeds in comparison to early dehydration. This is likely related to the time requirement for infection by *Pyrenophora semeniperda*, which microscopic observations show has spores that take approximately 6-8 hours to germinate under optimal conditions and 24 hours for mycelia to penetrate the caryopsis (H. Finch, unpublished data).

Pathogen activity varied as a function of dehydration water potential. The proportion of dormant and non-dormant seeds killed was highly dependent upon the interaction of all treatment variables. A dehydration water potential of -4 MPa is clearly above the threshold for critical



processes associated with pathogen growth, including conidial germination, infection, and mycelia growth within the seed. However, stomatal development is delayed until transfer to free water. Mortality of both dormant and non-dormant seeds dehydrated at -4MPa was high regardless of imbibition period because disease development could continue during dehydration. This is consistent with our earlier studies that showed the pathogen could progress during incubation at -2 MPa, with rapid stomatal production following transfer to water (Finch *et al.*, 2013).

A dehydration water potential of -10 MPa is apparently near the threshold for disease development. Results were equivocal when paired with early dehydration, while late dehydration at this water potential resulted in high seed mortality following a sufficient rehydration period. A dehydration water potential of -40 MPa may be below the threshold for conidial germination and infection; early-dehydrated seeds failed to develop stomata following rehydration. However, following late dehydration, mortality was directly related to dehydration duration, indicating that some pathogen-mediated process deleterious to the seed could take place *in vivo* at this water potential. A dehydration water potential of -150 MPa was apparently too low for pathogen growth. Its only effect on mortality was that rapid dehydration at this water potential following a 24-hour imbibition period apparently slowed the seeds down sufficiently to permit some mortality to occur. Mortality did not increase as a function of time in dehydration, indicating that the increase in mortality was not due to the effect of the pathogen during dehydration.

Seed dormancy status influences the outcome of the *Bromus-Pyrenophora* pathosystem; specifically, dormant seeds are nearly always killed by *P. semeniperda* because they lack the ability to escape through germination. Fully after-ripened (non-dormant) seeds are less likely to

be killed by the fungus because they can germinate before disease development is completed. While dormant seeds are most often killed by the fungus, mortality of dormant seeds in several treatments was reduced. Following infection, the fungus appeared to tolerate prolonged dehydration at all water potentials. If imbibed seeds dry before successful germination occurs the fungus may remain viable and even active inside the seed. We observed that inoculated seeds imbibed in water for 24 hours were more likely to be killed following dehydration and rehydration than seeds imbibed for only 8 hours. Because fungal spores require at least six hours to germinate, this observation is probably at least partially the result of infection failure.

Vertucci and Farrant (1995, Table 1) proposed five specific seed hydration levels that correspond to qualitative changes in metabolic activity. Seeds are capable of completing germination only at the highest hydration level. From -3 to -40 MPa, seeds experience unregulated catabolism, free radical production and enzymatic degradation. In our studies this is approximately the same range of water potentials where pathogenicity of *P. semeniperda* is highly favored. Retrieval of *B. tectorum* seeds from autumn seed bank samples demonstrates that the pathogen can survive inside seeds at the extremely low water potentials experienced during summer; when brought from the field and exposed to continuous hydration in the laboratory, retrieved seeds developed stromata within 1-3 days (S. Meyer, unpublished). The pathogen appears most likely to survive low water potentials if it has already successfully penetrated the seed caryopsis. Lower water potential limits for *P. semeniperda* disease development are similar to those reported for other fungi. For example, strains of *Aspergillus ochraceus* on barley grains were inhibited at water potentials lower than -30 MPa (Torres *et al.*, 2003). The range over which seed fungi are favored relative to the seeds they infect may vary

according to species but nonetheless represents a range of partial hydration that inhibits seed germination.

While germination outcomes are influenced by a variety of environmental variables (Meyer *et al.*, 1997; Allen, 2003; Allen *et al.*, 1993a; Vertucci and Farrant, 1995), water availability remains a factor of overriding importance in terms of pathogen-seed relations (Halloin, 1986). Water uptake during seed germination can be characterized as a triphasic pattern; rapid hydration that is largely a physical process is followed by a lag phase and a second phase of rapid hydration following radicle emergence (Bewley *et al.*, 2013). In many habitats, seeds may complete phases one and two multiple times due to intermittent dehydration (Debaene-Gill *et al.*, 1994; Allen *et al.*, 1993a; Meyer *et al.*, 1997; Christensen *et al.*, 1996).

Precipitation levels during the year may potentially result in a high degree of infection if *P. semeniperda* inoculum levels are high (Finch *et al.*, 2013). As indicated by levels of *Pyrenophora*-killed seeds retrieved from soil seed banks, seeds mature at a time when maximum inoculum levels of the fungus (i.e., spore-bearing stromata on killed seeds from the previous year) are present (Beckstead *et al.*, 2007; Meyer *et al.*, 2007). The *Bromus-Pyrenophora* pathosystem is influenced by water availability in the context of three fungal-seed interaction sequences (Finch *et al.*, 2013), associated with particular seasons of the year. During summer when seed populations are characterized by high primary dormancy, infected seeds across a range of water potentials may be killed by the fungus over a series of hydration-dehydration episodes. During autumn, infected seeds may escape death due to rapid germination following a germination-triggering rainfall event, or be killed if radicle emergence is delayed by exposure to low water potentials. During winter/spring, partially imbibed seeds that fail to germinate during autumn may become secondarily dormant and carry over in the seedbank. If previously infected

during autumn or winter, seeds may be killed during the spring or subsequent summer or fall. Thus each of the likely seasons of interaction permits infection, and in cases of fluctuating water availability the fungus nearly always causes high seed mortality.

Studies on perennial ryegrass (*Lolium perenne*) and *B. tectorum* seeds showed that seeds of these grasses progressed toward radicle emergence and were not killed or rendered dormant when hydration was repeatedly interrupted by dehydration episodes at -4 or -10 MPa, but at water potentials lower than -40 MPa, germination was substantially delayed when dehydration was initiated near the onset of radicle emergence (Allen *et al.*, 1993a; Debaene-Gill *et al.*, 1994; Allen *et al.*, 1993b). While the present study illustrates that infection and disease development clearly occur during hydration-dehydration sequences, a clear understanding of how the *Bromus-Pyrenophora* pathosystem is affected by low water potentials (i.e., <-150 MPa) frequently observed in dry soils during summer is still incomplete. For example, we do not know whether seeds initially infected while seeds are dormant can escape the fungus through rapid germination after seeds lose dormancy through after-ripening in summer.

The importance of low water potentials characteristic of semi-arid soils are too often overlooked in seed-fungal pathosystems; variable temperatures, water evaporation from soils, and precipitation all contribute to widely fluctuating water potentials (Bair *et al.*, 2006; Meyer and Allen, 2009). Under measured field conditions, *B. tectorum* seeds regularly experienced water potentials that are highly favorable to *P. semeniperda* (e.g. -4 to -40 MPa). However, these intermediate water potentials are often experienced for just a few hours because soils are either wetting during a precipitation event, or drying following a precipitation event (Meyer and Allen, 2009). During the summer, field water potentials in dry soils can fluctuate between -150 and -800 MPa in dry soils (Bair *et al.*, 2006; Meyer and Allen, 2009).

Because disease development occurs at water potentials lower than those that support rapid progress toward completion of germination, *P. semeniperda* gains advantage under most hydration-dehydration scenarios we have tested. In terms of field-realistic conditions, infected after-ripened seeds subjected to intermittent hydration are more likely to be killed than to germinate. Initially dormant and secondarily dormant seeds are less likely to be killed due to the dormancy breaking effects of intermittent hydration that induce germination. Long initial imbibition also increases the likelihood of seed mortality; if inoculated seeds are subjected to precipitation that is insufficient in causing germination but results in successful infection, following dehydration and rehydration events will result in a larger proportion of *P. semeniperda*-killed seeds. To our knowledge, this is the first time a seed-fungal pathosystem has been explored in the context of widely fluctuating water availability to seeds. In these scenarios, disease development can continue at low water potentials that restrict seed radicle emergence, explaining how fluctuating water availability across a range of water potentials favors the fungus and therefore results in high mortality of *B. tectorum* seeds.

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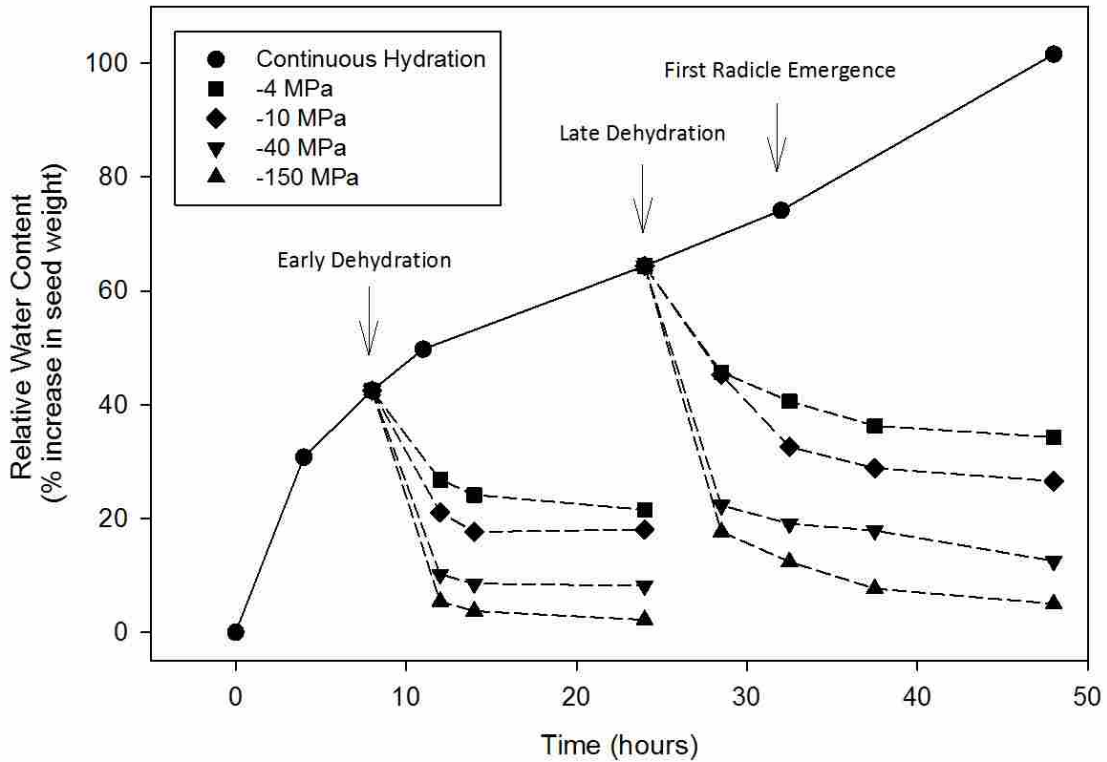
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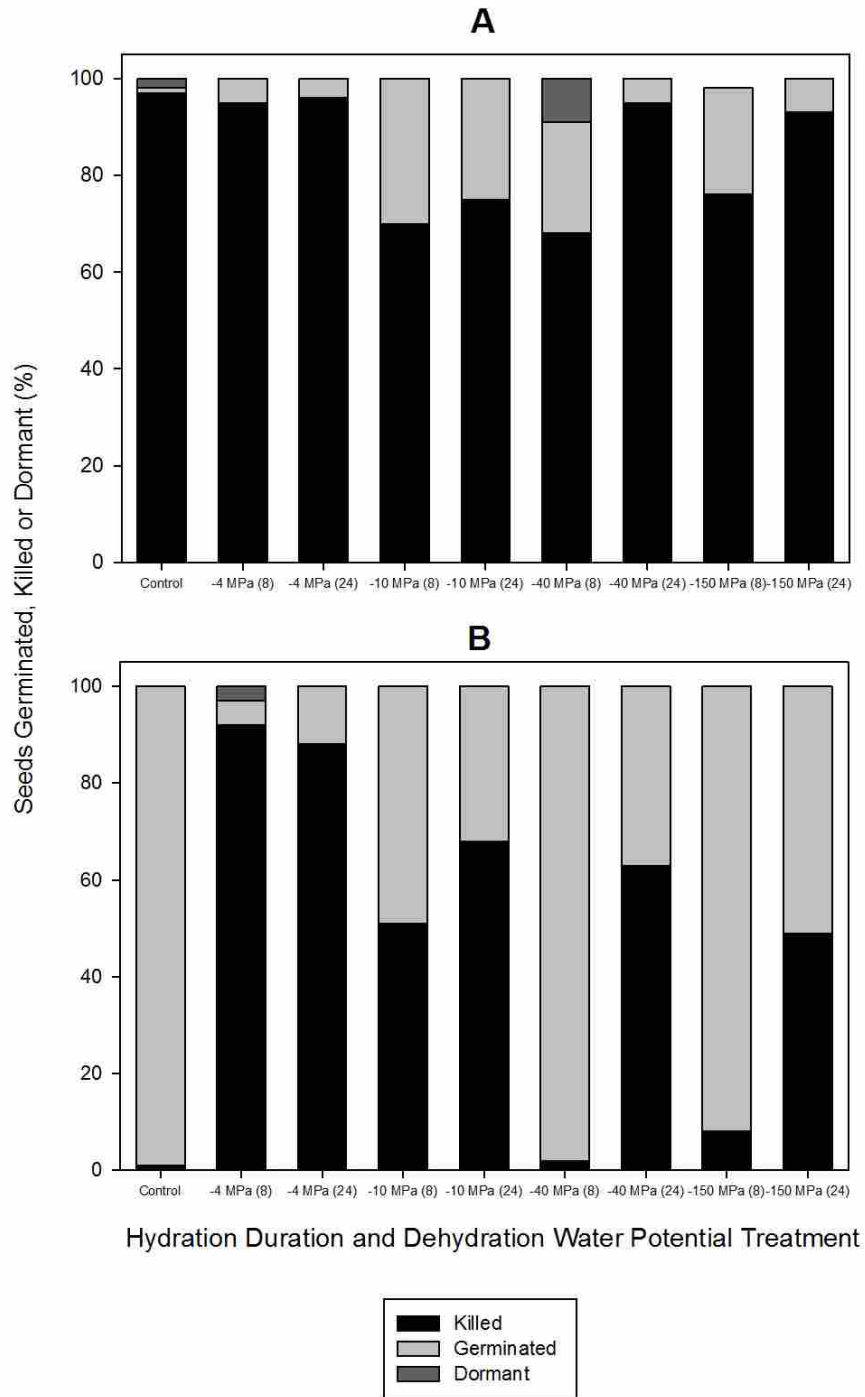
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## FIGURES

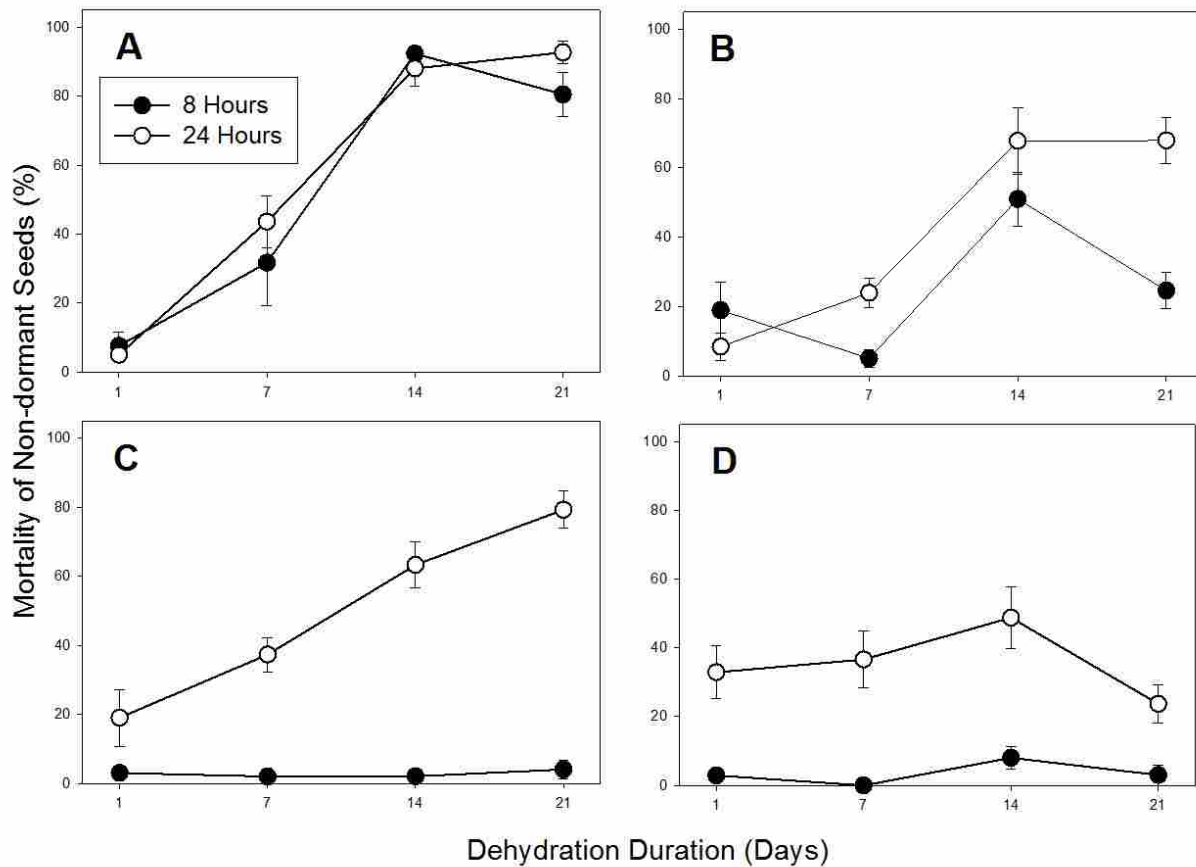


**Figure 1. Water Content of Non-dormant *Bromus tectorum* Seeds when Hydrated or Dehydrated.**

Water content of non-dormant *Bromus tectorum* seeds hydrated continuously (solid line) or dehydrated at indicated water potentials following hydration for 8 or 24 hours (dashed lines). Early Dehydration was timed to begin approximately when the rapid phase of imbibition was completed, while Late Dehydration was timed to occur shortly before the first radicle emerged. Dormant seeds fail to achieve a relative water content above 60% (not shown). Incubation temperature was 20°C throughout. Under continuous hydration, fungal stromata do not begin to appear until 11 days, so continuously hydrated non-dormant seeds escape death even if they become infected.

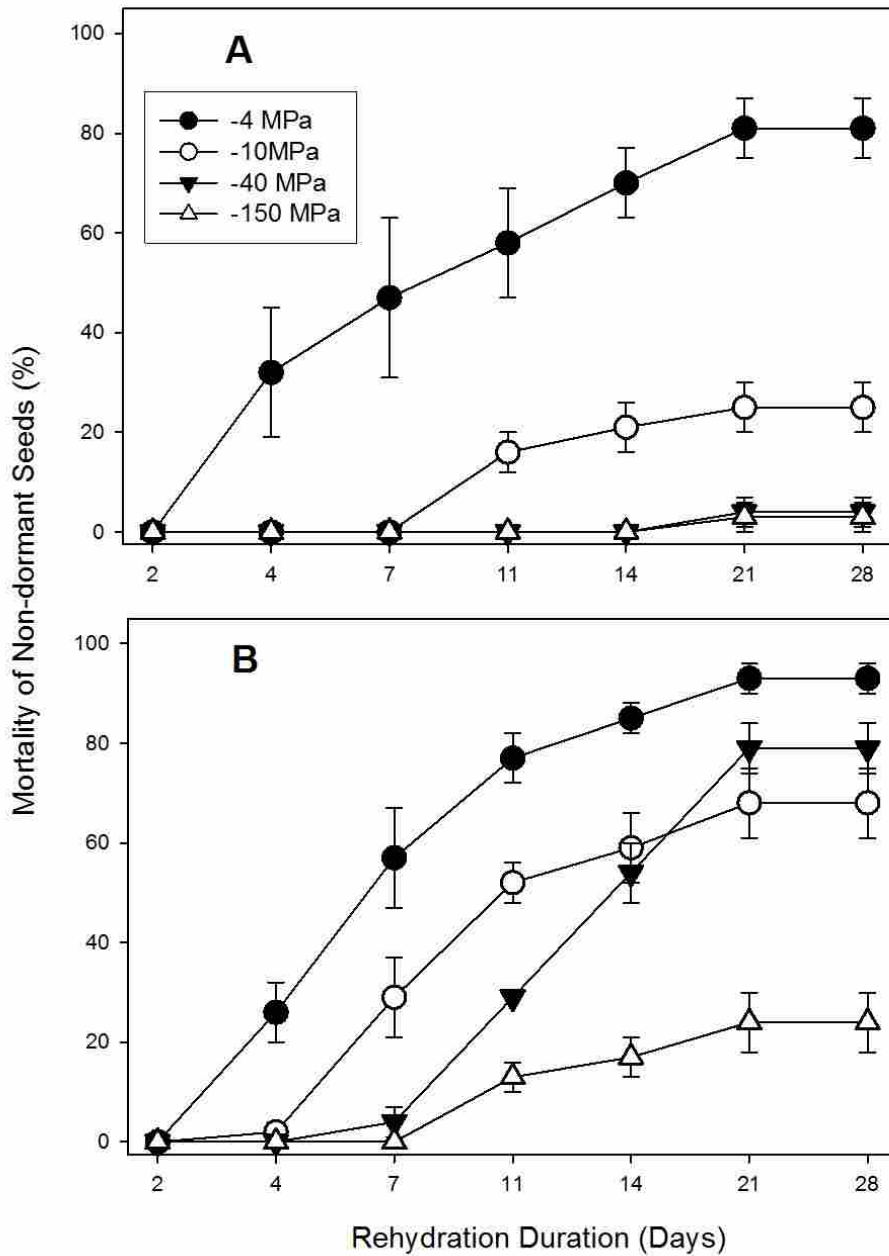


**Figure 2. Mortality of Dormant and Non-dormant Seeds When Hydrated, Dehydrated and Rehydrated.** Outcomes of hydrated-dehydrated-rehydrated initially dormant (A) and non-dormant (B) seeds from the same population. *Bromus tectorum* seeds were imbibed (20°C) in the presence of *Pyrenophora semeniperda* for either short (8 hours) or long (24 hours) hydration periods then dehydrated at water potentials of -4, -10, -40, or -150 MPa for 14 days, followed by rehydration. Control treatments received continuous hydration in water.



**Figure 3. Mortality of Non-dormant *B. tectorum* Seeds Subjected to Hydration-Dehydration-Rehydration Treatments in the Presence of *P. semeniperda*.**

Seeds were imbibed for short (8 hours) or long (24 hours) periods, then dehydrated at water potentials of -4 “A”, -10 “B”, -40 “C”, or -150 “D” MPa for a duration of 1, 7, 14, or 21 days, then rehydrated in water for 28 days. Bars represent the standard error of the mean.



**Figure 4. Mortality Time Curves of Non-dormant Seeds Upon Rehydration.**

Mortality of non-dormant *B. tectorum* seeds subjected to hydration in the presence of *P. semeniperda* for 8 hours (“A”), or 24 hours (“B”) and dehydration water potential treatments (-4, -10, -40, or -150 MPa) for 21 days, followed by 28 days of rehydration. Bars represent the standard error of the mean.

### Chapter 3

The Characterization of the Asexual Disease Life Cycle of *Pyrenophora semeniperda* on a  
*Bromus tectorum* Seed Using Scanning Electron Microscopy

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### ABSTRACT – ARTICLE 3

While leaflet and pre-dispersal infection of grass seeds by *P. semeniperda* have been previously characterized (Medd *et al.*, 2003; Campbell and Medd 2003; Medd and Campbell, 2005), infection of mature seeds following dispersal remains an area of significant importance that lacks detailed characterization. This study was conducted to expand and clarify the disease cycle presented by Medd and others (2003), in addition to providing an accurate disease development cycle of the asexual state of *P. semeniperda* when mature seeds are challenged with this pathogen. Dormant *B. tectorum* seeds were inoculated with *P. semeniperda* and continuously hydrated for periods of 6 hours to 21 days. Samples were chemically fixed and viewed with a scanning electron microscope. *Pyrenophora semeniperda* spores germinate within 6-8 hours. Hyphae randomly grow across the surface of the seed and produce mucilage. Appressoria form on the ends of hyphae and also penetrate the seed through openings such as stomata and broken trichomes. Following eight days of disease development, the endosperm collapses as it is consumed by the fungus. Within 11-14 days, seeds develop fungal stromata. Stromata bear conidiophores following penetration from the seed. Within 21 days, conidia were produced and most commonly observed in pairs, morphologically resembling a “Y”. These data ultimately expand, clarify, and correct the putative disease cycle presented by Medd and others (2003), in addition to providing detailed images of the asexual disease development life cycle of *P. semeniperda* on seeds.

Keywords: *Pyrenophora semeniperda*, *Bromus tectorum*, pathosystem, seeds, disease development, asexual, life cycle.

## INTRODUCTION

The ascomycete fungus *Pyrenophora semeniperda* has been considered a weak pathogen that infects seeds and causes leaf spotting of at least 35 genera of annual and perennial grasses (Campbell and Medd, 2003; Medd *et al.*, 2003). The fungus is generally observed as *Drechslera campanulata*, the anamorph state (Medd *et al.*, 2003), 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).

Medd and colleagues (2003) proposed a putative disease life cycle for *P. semeniperda* that included leaf infection of seedlings, resulting in ring spots, and also infection of developing seeds (referred to as floral infection). Post-dispersal-infection of mature seeds (as would occur in seed banks) was deemed possible, but unlikely to result in seed death. Rather, infection resulted in a temporary reduction of seedling growth (Wallace, 1959; Barreto and Fortugno, 1994; Campbell and Medd, 2003). Recent studies have shown that *P. semeniperda* causes the death of mature seeds that have been dispersed from the maternal plant and also the death of post-dispersal-infected seeds (Beckstead *et al.*, 2007; Meyer *et al.*, 2007; Meyer *et al.*, 2008). In fact, the fate of seeds infected by *P. semeniperda* is largely a function of their germination rate and water availability within soils (Beckstead *et al.*, 2007; Finch *et al.*, 2013a).

Mature, dormant (Meyer *et al.*, 2007) and non-dormant (Finch *et al.*, 2013a) seeds of the annual grass weed *Bromus tectorum* can also suffer high mortality in field seed banks following exposure to soilborne *P. semeniperda* inoculum (Meyer *et al.*, 2007). This knowledge contrasts the disease life cycle presented by Medd and others (2003); earlier work focused on *P. semeniperda* as a pre-dispersal fungus (florete infection) when significant seed mortality occurs post-dispersal (Meyer *et al.*, 2008).



Leaflet and pre-dispersal seed infection by *P. semeniperda* have been actively characterized (Medd *et al.*, 2003; Campbell and Medd 2003; Medd and Campbell, 2005). However, infection following post-dispersal remains an area of significant importance that lacks detailed characterization, thus the overall objective of our study was to elucidate the pathosystem interactions of *B. tectorum* and *P. semeniperda* in mature seeds using scanning electron microscopy (SEM) to address the following questions: (1) what are the morphological processes of the pathogen that occur on a dormant *B. tectorum* seed under continuous hydration as observed by SEM, (2) is there evidence of appressorial formation and penetration similar to that observed on leaves (Campbell and Medd 2003), and (3) what similarities and differences exist between floret and leaf infection compared to post-dispersal seed infection. This fundamental knowledge will expand and clarify the disease cycle presented by Medd and others (2003), in addition to providing detailed disease development cycle of the asexual state of *P. semeniperda* when mature seeds are challenged with this pathogen.

## **MATERIALS AND METHODS**

### **Seed Population and Fungal Inoculum**

Mature caryopses (hereafter seeds) of *B. tectorum* L. were collected from a wild population at the Brigham Young University Research Farm (Spanish Fork, Utah, USA) in June 2011. The chafe of seeds was removed by hand and seeds were stored in a -10°C freezer to maintain a condition of primary dormancy. The *P. semeniperda* inoculum originated as a moderately virulent strain collected from Whiterocks, Utah, USA, and was produced as described by Meyer *et al.*, 2010.

### **Seed Inoculation and Sampling**

Approximately 100 seeds were inoculated with either one or multiple spores. Prior to single spore inoculation, *P. semeniperda* spores were placed on a polycarbonate membrane and hydrated for 20 minutes with distilled water. Using the end of a 2 mm diameter glass probe (made into a needle by burning the ends of two glass probes that face one another, then quickly pulling them apart while the glass was hot and malleable), individual spores were picked up with the needle under a light microscope and placed on a seed. Seeds were inoculated with multiple spores by placing them in a vial of approximately 3 mg of spores and 1 ml of distilled water, and shaking for 10 seconds.

All inoculated seeds were placed on a polycarbonate membrane that was laid on top of two water-saturated blue germination blotters (Anchor Paper, St. Paul, Minnesota, USA) inside a 100 mm Petri dish. Covered dishes were placed in plastic sleeves and incubated with cycles of 12 h fluorescent light/12 h dark at approximately 20°C, which is near optimum for the pathogen (Campbell *et al.*, 1995). Seeds were sampled at periods of 6 hours to 28 days post-inoculation.

### **Additional Sampling**

Additional images were taken of approximately 50 dormant *B. tectorum* seeds that were subjected to one of the following treatments prior to inoculation: 1) removal of the lemma and palea (naked caryopsis), 2) autoclaved (dead) and dried for 24 hours, 3) surface sterilized by immersing seeds for one minute in ethyl alcohol (70%) followed by one minute in bleach (10%), or two minutes in ethyl alcohol followed by two minutes in bleach, 4) removal of the lemma and palea followed by either short or long surface sterilization as described, and 5) removal of the lemma and palea followed by autoclaving. Surface-sterilized seeds were rinsed with sterile distilled water before being inoculated.

## Specimen Preparation for SEM

Following incubation, samples were freeze dried or chemically fixed. Chemically fixed samples were placed directly in primary fixative or first frozen in liquid nitrogen and fractured into small pieces prior to being placed in the fixative (primary fixative was a 2% glutaraldehyde solution buffered with sodium cacodylate at pH 7.3). Samples were washed in cacodylate buffer, secondarily fixed and stained with 1% osmium (OsO<sub>4</sub>) solution buffered with sodium cacodylate at pH 7.3, then washed with distilled water. Samples were then dehydrated with a graded series of acetone and critical point dried (CPD). All samples were coated with gold/palladium and evaluated with a scanning electron microscope (model FEI XL30 ESEM FEG).

## RESULTS

Conidia spores of *P. semeniperda* were multiple-celled and cylindrical in shape. Conidia ranged from 30-100µm in size and were most commonly observed to germinate from one or both polar cells (Fig. 1A). While germination from intermediate cells was not observed in our study, it has been viewed with a light microscope (S. Clements, unpublished data). Germination of hydrated conidia commenced within 6-8 hours. Following germination, conidia produced germ tubes or hyphae that branched (Fig. 1B) and were long and slender in appearance, giving rise to several penetration sites. Hyphae grew in random directions across the surface of the seed and were observed to produce mucilage within 12 hours post-inoculation. Both mycelium and mucilage gradually covered the seed over the course of disease development (Fig. 1C).

Hyphae penetration into the seed was observed within 24 hours following inoculation. Appressoria formed on the seed surface at the tip of hyphae, and morphologically resembled swollen hyphae or were club-shaped. They were observed to penetrate the lemma (Fig. 1D),

palea, and stomata (Fig. 1E). Hyphae were also observed to penetrate cracked surfaces in the lemma (Fig. 1F) and palea, broken trichomes (Fig. 2A), and the abscission layer (Fig. 2B) directly. Following penetration mycelium appeared to grow back outside of seeds containing both the lemma and palea. Mycelium repeatedly penetrated into and exited out of the seed, resulting in a visible network of mycelium on the surface.

Fractured seeds sampled 8 days post-inoculation revealed a hollow center void of endosperm tissue (Fig. 2C). Early stage fungal stromata exited the caryopsis within 11-14 days and bore conidiophores immediately following protrusion (Fig. 2D). All stromata did not form within the same time frame; some stromata appeared within 11 days, while others appeared within 12-16 days. This resulted in both stromata and conidiophores that varied in height and/or stage of development (Fig. 2E). As stromata grew longer, conidiophores became more numerous (Fig. 2F). Increased height caused stromata to be more fragile and easily broken. Stromata were observed to grow as long as 4 mm.

Prior to conidia formation, conidiophores appeared to be darkly segmented within the length of the conidiophore (Fig. 3A). Production of conidia was observed to occur following 21 days of disease development. As conidia formed, they became pinched at the ends of conidiophores, and were most commonly observed to form in pairs. Conidia on the ends of conidophores were usually observed to have the morphology of a “Y” (Fig. 3B).

Additional samples subjected to treatments such as lemma and palea removal, autoclaving, and surface-sterilization indicated that disease developed similarly to continuously hydrated dormant seeds. However, the onset of disease development was accelerated following lemma and palea removal and surface-sterilization treatments; dramatic acceleration of disease

development was observed in seeds that were autoclaved prior to inoculation. In this case, seeds developed fungal stromata within 5-6 days (data not shown).

## DISCUSSION

This study presents a developmental account of mature *B. tectorum* seeds challenged with *P. semeniperda*. It describes the events that commence between conidial inoculation and conidial production. Our observations have allowed us to expand and clarify the known disease life cycle presented by Medd and others (2003) by accurately characterizing the asexual disease life cycle of post-dispersal infection on a mature *B. tectorum* seed (Fig. 4).

Spores of the pathogen strain used in this study were observed to germinate as early as 5 hours following imbibition in water. Similar to *P. teres* (Van Caesele and Grumbles, 1979; Coyle and Cooke, 1993), *P. graminea* (Smedegård-Petersen, 1976), *P. avenae* (Arora *et al.*, 1980) and *P. dictyoides* (Cromey and Cole, 1985), *P. semeniperda* germ tubes have been observed to arise from polar and intermediate cells, but never simultaneously from both. Previous studies reported that some spores of this fungus germinate within 3 hours of incubation (Campbell and Medd 2003), while most isolates commonly used in our virulence trials germinated within 6 hours (Meyer *et al.*, 2010). Campbell and Medd (2003) reported that conidia averaged 16 $\mu$ m in length. Our conidia were significantly larger in size, ranging from 30-100 $\mu$ m.

Following production of mycelium, a mucilaginous matrix was observed to coat the surface of the seed. In studies on spore attachment of pathogens to plants (Jones and Epstein 1989; Howard *et al.*, 1991; Tucker and Talbot 2001; Zhu *et al.*, 2013), adhesion of fungal spores to the plant surface often occurs due to mucilage. Additionally, mucilage may be required for

host recognition and subsequent fungal development (Tucker and Talbot, 2001). Mycelium was also observed to wrap around trichomes, further adhering spores to the plant. Seeds infected earlier than 8 hours were not observed with the SEM, likely because spores had not adequately adhered themselves to the seed prior to chemical fixation, or had failed to germinate.

Although we consider *P. semeniperda* as an opportunistic fungus due to its ability to penetrate numerous structures and openings, stomata penetration did not always take place when hyphae were near guard cells. Hyphae were observed to either pass by or grow over guard cells of stomata without entering them. This phenomenon was observed for both open and closed stomata, and is consistent with observations of other plant fungi (Wong *et al.*, 2012; Babu *et al.*, 2007; Campbell and Medd 2003).

Appressorial formation and penetration on leaves and mature seeds was found to be similar. It was reported by Campbell and Medd (2003) that appressoria were often produced over epidermal cell wall junctions, epidermal cells, stomatal guard cells, and trichomes. We found appressorial formation and/or penetration to occur within these same structures in the seed, in addition to the abscission layer.

While the exact time of penetration was difficult to determine, it was observed to occur within 24 hours following inoculation. Related studies on the *B. tectorum*-*P. semeniperda* pathosystem have shown that mycelium is more likely to survive severe dehydration events if inoculated seeds are imbibed for a period of 24 hours prior to dehydration. Seeds imbibed for only 8 hours were much less likely to be killed following dehydration (Finch *et al.*, 2013b, in review). Because penetration was observed to occur within 24 hours, and higher mortality of seeds following imbibition for 24 hours and dehydration was observed, it is likely that mycelium

are highly desiccation tolerant once inside the seed (Finch *et al.*, 2013; Finch *et al.*, 2013b, in review).

Similar to its relatives, which cause foliar diseases of cereal crops (e.g. *P. tritici-repentis*, *P. teres*), *P. semeniperda* is a necrotroph (Meyer *et al.*, 2010). These particular pathogens invade host tissue by secreting toxins that create a front of dead tissue which is then digested enzymatically to provide nutrition to the fungus. *Pyrenophora semeniperda* has been shown to produce toxins, including cytochalasin B and other more unusual cytochalasins (Evidente *et al.*, 2002; Capio *et al.*, 2004). Within eight days, the endosperm center appeared to be hollow. Studies using light microscopy showed that starch granules were consumed, causing cells to collapse (Finch-Boekweg, unpublished data). It is unknown whether the fungus digests starch from cells in the center of the endosperm, or whether cells are consumed on the outside edge of the endosperm causing inward cells to move toward the outside aleurone layer; this is a subject for further investigation.

Within 11-14 days, macroscopic stromata were visible on the outside of the caryopsis. Early stages of stromata formation were unclear; whether the lemma and palea were parted or fractured as stromata began to protrude from the caryopsis is unknown. While conidiophore production was observed immediately following stromata appearance, conidia were not produced for an additional 7 days following stromatal formation. Production of conidia on the ends of conidiophores was most commonly observed in pairs.

Similarities of floret and leaf infection and post-dispersal infection of seeds included the random growth of germ tubes across the sample surface, appressorial morphology, and appressorial formation and penetration (Campbell and Medd, 2003). While it was reported that

more appressoria formed on seedling leaves than adult leaves, we observed that appressorial structures were commonly observed on mature seeds, but not required for penetration.

Differences of floret and leaf infection in comparison to post-dispersal infection included the collapse of host cells prior to contact with infection hyphae, the lack of endosperm or embryo mycelium development during floret infection, and the infection of a seed during anthesis resulting in no apparent detrimental effect on seed development (Campbell and Medd, 2003). Post-dispersal-inoculation of mature seeds showed that collapse of host cells occurred following infection, rather than prior to infection. This was evident by the collapse of cells within the endosperm following 8 days of disease development. While the endosperm and embryo of developing ovaries appeared to lack mycelium following 48 hours (Campbell and Medd, 2003), the influence of mycelium was highly evident within the endosperm following 8 days of disease development in a mature seed, and has been observed in the embryo as well (H. Finch, unpublished data). Infection of seeds during anthesis resulted in no apparent detrimental effect on seed development. In contrast, we found that mature seeds that are inoculated post-dispersal are found to develop fungal stromata within 11-14 days and lack the ability to germinate (indicating seed death). It is also interesting to note that autoclaved seeds obtained fungal stromata within 5-6 days, suggesting that living mature seeds have defenses that delay disease development.

The use of SEM is advantageous due to the higher resolution and depth of field. While good images were obtained using chemical fixation, sample preparation techniques caused some imperfection of conidia and mycelium. The freeze drier was used in an attempt to preserve samples, and allowed us to obtain better images of conidia, while other samples were only chemically fixed. The present study clearly showed the morphological interactions of *P*.



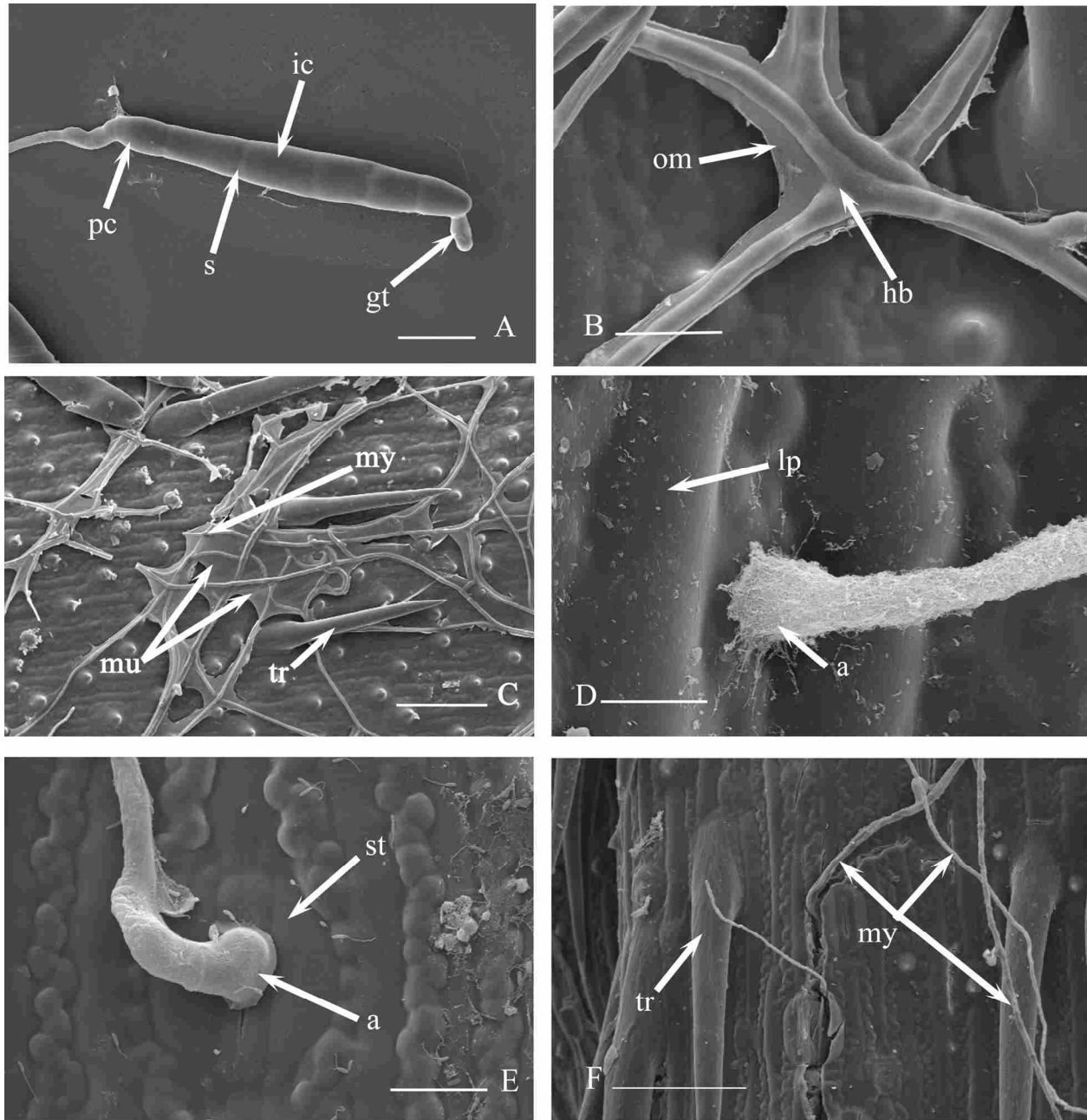
*semeniperda* and *B. tectorum* seeds. It was shown that appressorial formation and penetration is similar to that reported by Campbell and Medd (2003) on leaves, and that similarities and differences exist between floret and leaf infection, and post-dispersal infection of mature seeds. These data ultimately expand, clarify, and correct the putative disease cycle presented by Medd and others (2003), in addition to providing accurate images of the asexual disease development life cycle of *P. semeniperda*.

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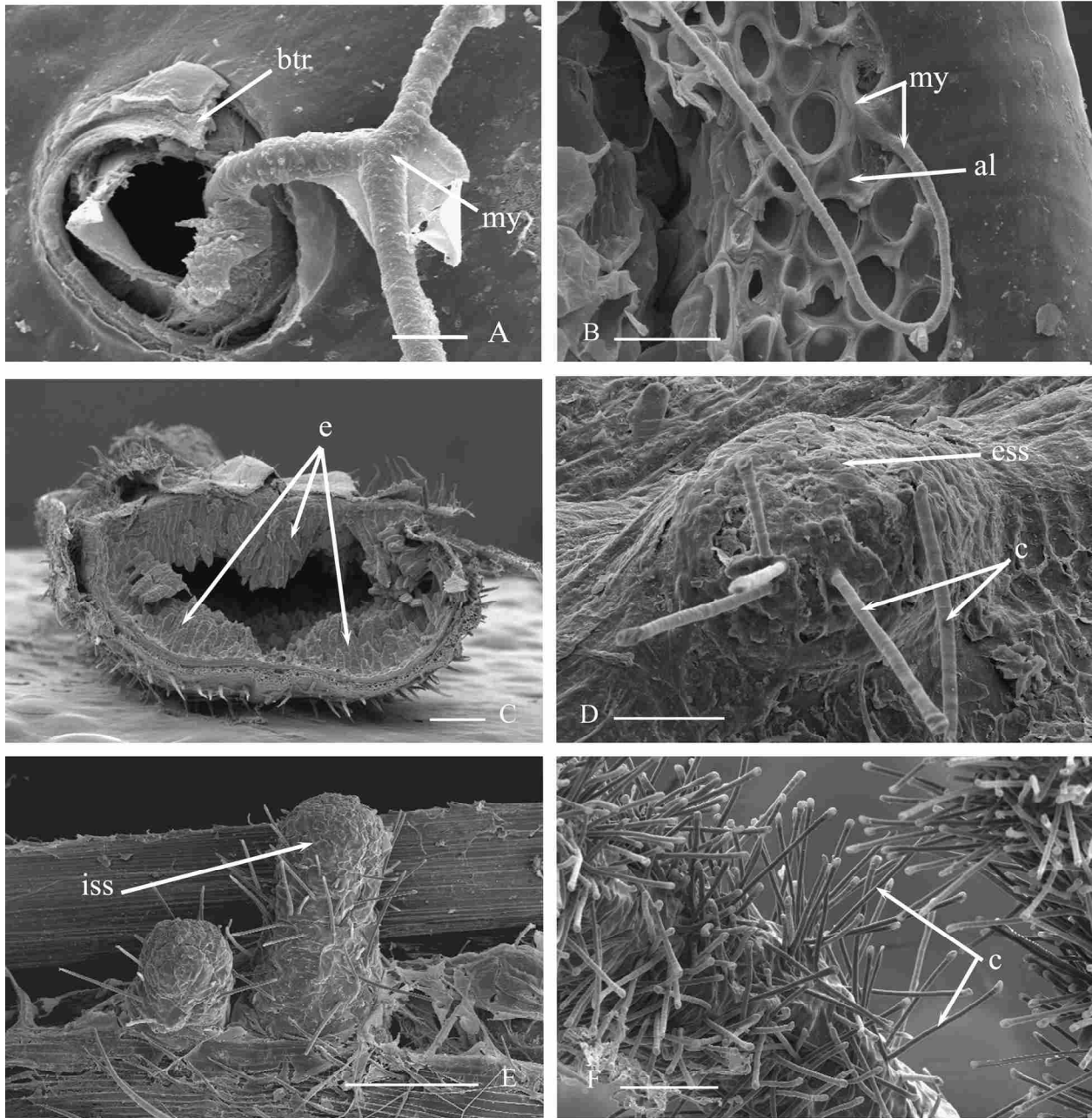
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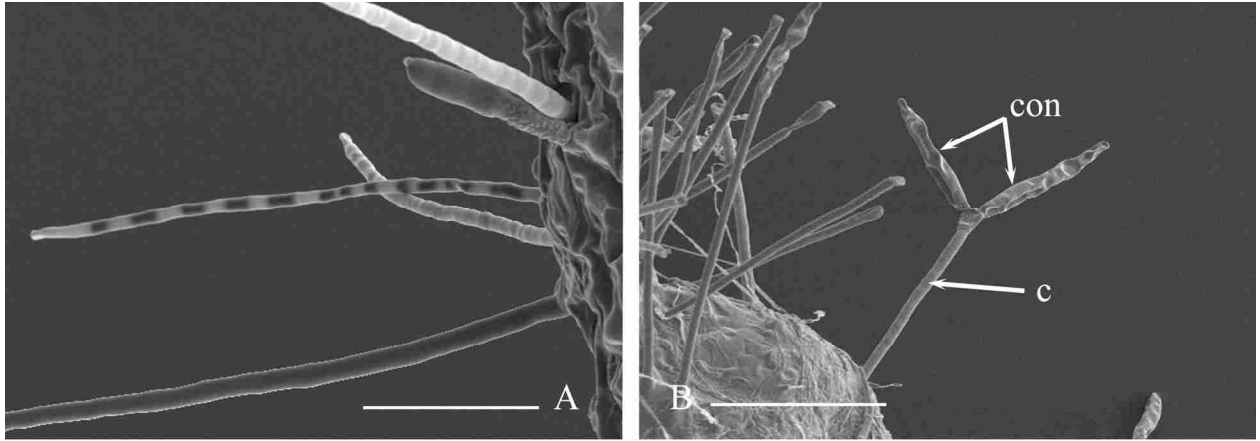
**Figure 1. Disease Development of *Pyrenophora semeniperda* On a Mature *Bromus tectorum* Seed.**

Dormant *Bromus tectorum* seeds were infected with *Pyrenophora semeniperda*. SEM images were taken of the disease cycle. Images are taken of the following: (A) germinated conidium, with septum (s) separating intermediate cells (ic) and germ tubes (gt) exiting polar cells (pc), (B) hyphal branching (hb) with the onset of mucilage (om), (C) growth of mycelium (my) and production of mucous (mu) around trichomes (tr), (D) appressorium (a) penetrating into the lemma or palea (lp), (E) appressorium (a) penetrating a stomata (st), and (F) mycelium (my) growing in and out of a crack in the lemma or palea near a trichome (tr). Bars indicate A-20 $\mu$ m, B-10 $\mu$ m, C-50 $\mu$ m, D-5 $\mu$ m, E-10 $\mu$ m, and F-50 $\mu$ m.



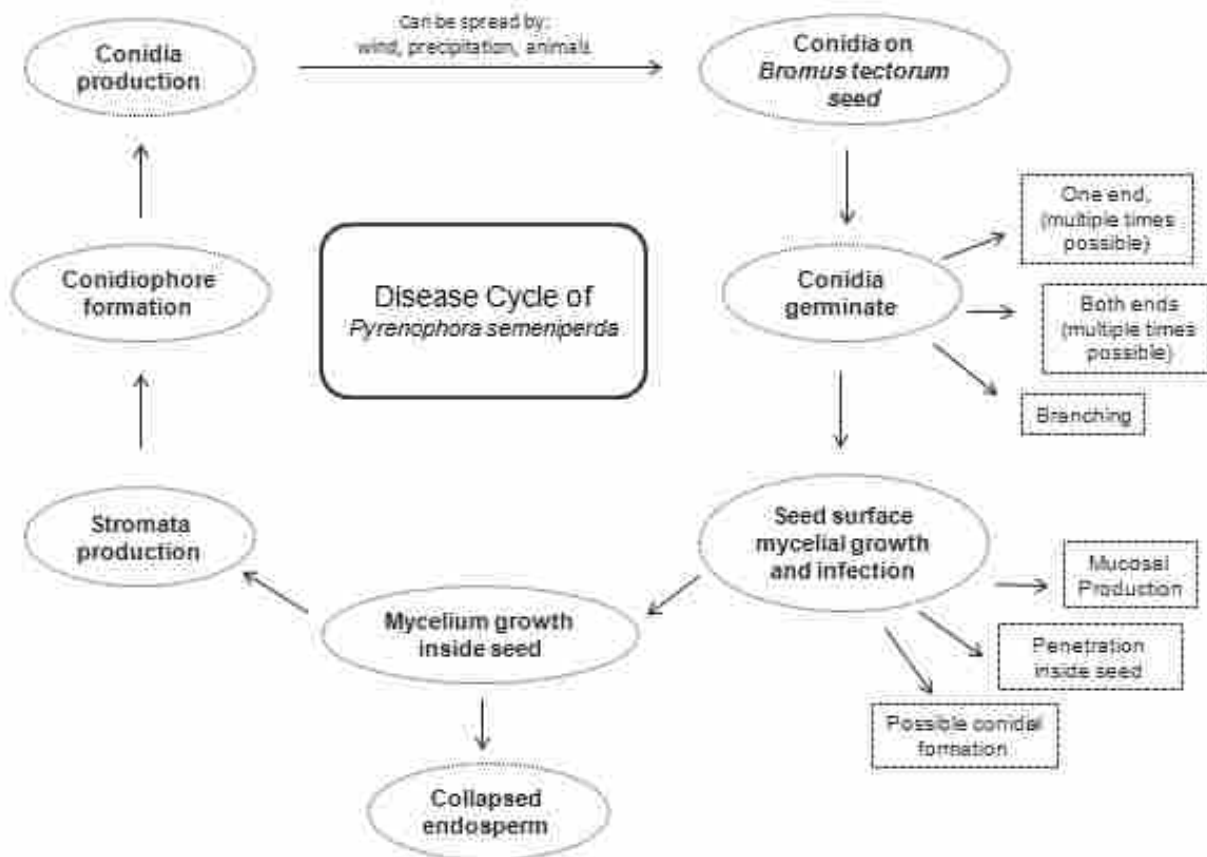
**Figure 2. Disease Development of the *P. semeniperda*-*B. tectorum* Pathosystem.**

Dormant *Bromus tectorum* seeds were infected with *Pyrenophora semeniperda*. SEM images were taken of the disease cycle. Images were taken of the following: (A) mycelium (my) penetrating a broken trichome (btr), (B) mycelium (my) penetrating the abscission layer (al), (C) a collapsed endosperm (e) that has been consumed by the fungus, (D) early stage development of stromata (ess) and production of conidiophores (c), (E) intermediate stage development of stromata (iss) protruding from the caryopsis, and (F) conidiophores (c) on a stromata. Bars indicate A-5 $\mu$ m, B-20 $\mu$ m, C-200 $\mu$ m, D-50 $\mu$ m, E-200 $\mu$ m, and F- 100 $\mu$ m.



**Figure 3. Disease Development of *P. semeniperda*.**

Dormant *Bromus tectorum* seeds were infected with *Pyrenophora semeniperda*. SEM images were taken of the disease cycle. Images were taken of the following: (A) a darkly segmented conidiophore prior to production of conidia, and (B) production of conidia (con) in pairs on a conidiophore (c). Bars indicate A- 50 $\mu$ m, and B-100 $\mu$ m.



**Figure 4. The Asexual Disease Cycle of *Pyrenophora semeniperda* on a Mature *Bromus tectorum* Seed.**

Dormant mature *Bromus tectorum* seeds were inoculated with *Pyrenophora semeniperda* following dispersal. The asexual disease life cycle was characterized with SEM. SEM images indicate that when seeds come in contact with conidia, if hydrated, conidia germinate at one or both polar cells. Following germination, hyphae branch and produce mucous. Hyphae may penetrate the caryopsis and grow on the surface of the seed. Mycelium growth occurs inside the seed, consuming cells and causing the collapse of the middle of the endosperm (Finch, 2013). Stromata are produced bearing conidiophores following protrusion from the caryopsis. Conidia are produced on the ends of conidiophores, and break off following completed development. Spores can be spread by wind, precipitation, and animals (Meyer *et al.*, 2008).



## APPENDIX A: ARTICLE 4

Disease Development of *Pyrenophora semeniperda* inside a *Bromus tectorum* Seed:

A Closer Look at the Embryo and Endosperm

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## INTRODUCTION

*Pyrenophora semeniperda*, an ascomycete that attacks grass seeds, has been established as a seed bank pathogen and has been observed to infect and kill dormant and non-dormant *Bromus tectorum* seeds (Meyer *et al.*, 2007; Finch *et al.*, 2013). While *P. semeniperda* was earlier reported to only cause leaflet and pre-dispersal infection of seeds (Medd *et al.*, 2003), studies have shown that *P. semeniperda* can infect seeds following dispersal (Beckstead *et al.*, 2007; Meyer *et al.*, 2007; Meyer *et al.*, 2008; Meyer *et al.*, 2010; Finch *et al.*, 2013, Finch 2013). The putative disease cycle presented by Medd and colleagues (2003) has been expanded and clarified (Beckstead *et al.*, 2007; Meyer *et al.*, 2007; Beckstead *et al.*, 2012; Finch, 2013), leading to further knowledge of disease development on the surface of a *B. tectorum* seed.

Campbell and Medd (2003) previously inoculated developing seeds on a plant and found that hyphae were not observed within the embryo or endosperm following 48 hours post-inoculation. However, images obtained with scanning electron microscopy (SEM) clearly showed that mycelium consumes the endosperm of a mature dormant *B. tectorum* seed within 8 days of disease development. *Pyrenophora semeniperda* infection of *B. tectorum* seeds following post-dispersal does occur (Beckstead *et al.*, 2007; Meyer *et al.*, 2007; Finch *et al.*, 2013; Finch, 2013) and is of further interest.

Based on earlier anatomical characterization of mature wheat and corn kernels, (Bradbury *et al.*, 1956; Wolf *et al.*, 1952), we sought to characterize the morphological changes caused by *P. semeniperda* on *B. tectorum* grass caryopses (hereafter “seeds”). Using light microscopy, the objective of this study was to characterize disease development of *P. semeniperda* at days 3, 8, and 14 post-inoculation within the endosperm and embryo of infected *B. tectorum* seeds, providing additional knowledge to the corrected disease cycle presented by Finch (2013). While

*P. semeniperda* has been established as a necrotroph (Meyer *et al.*, 2010), this additional knowledge will lead to further understanding of how the fungus attacks and ultimately kills seeds.

## **METHODS**

### **Seed Population and Fungal Inoculum**

Seeds of *B. tectorum* L. were collected from a wild population at the Brigham Young University Research Farm (Spanish Fork, Utah, USA) in June 2011. Seeds were cleaned by hand and stored in a -10°C freezer to maintain seeds in the primary dormant condition. The *P. semeniperda* inoculum originated as a moderately virulent strain collected from Whiterocks, Utah, USA, and was produced as described by Meyer *et al.*, 2010.

### **Seed Inoculation and Sampling**

Approximately 75 seeds were inoculated with multiple spores by placing them in a vial of approximately 3 mg of spores and 1 ml of distilled water, and shaking for 10 seconds. All inoculated seeds were placed on the surface of a polycarbonate membrane on top of two water-saturated blue germination blotters (Anchor Paper, St. Paul, Minnesota, USA) inside a 100 mm Petri dish. Dishes were placed within plastic sleeves and incubated with cycles of 12 h fluorescent light/12 h dark at approximately 20°C, which is near optimum for the pathogen (Campbell *et al.*, 1995) for periods of 3, 8, and 14 days.

### **Specimen Preparation for Cross Sectioning and Light Microscopy**

Following 3, 8, or 14 days, seeds were removed from the Petri dish and placed in liquid Nitrogen for approximately 20 seconds. Seeds were fractured and placed in primary fixative (2% glutaraldehyde solution buffered with sodium cacodylate at pH 7.3) for approximately 2-3 days. Following fixation, samples were washed in cacodylate buffer and secondarily fixed and

stained with 1% osmium (OsO<sub>4</sub>) solution buffered with sodium cacodylate at pH 7.3. Samples were then washed with distilled water and dehydrated with a graded series of acetone.

Following secondary fixation, samples were embedded in Spurr's resin, creating plastic sample-containing capsules. Capsules were trimmed and cross and longitudinally-sectioned to approximately 1µm thick sections using a diamond knife. Following cross-sectioning, samples were dyed blue and placed on microscope slides to be viewed with a light microscope.

## RESULTS

Following three days of disease development, cross sections of the endosperm and embryo revealed that the seed tissues appear relatively un-affected by the fungus. Penetration into seeds containing both the lemma and palea has been observed to occur within 24 hours (Finch, 2013). Small amounts of mycelium are detectable inside the seed following 3 days. A complete view of the endosperm reveals compacted and elongated cells filled with starch granules (Fig. 1A). Most are arranged end to end with long axes radiating in all directions from the vertical fissure (Fig. 1B). The smallest endosperm cells are observed directly below the aleurone layer (Fig. 1C). The living aleurone cells lack visible starch granules and are known to support a heavy growth of fungi when the seed coat is penetrated (Wolf *et al.*, 1952). The testa lies above the aleurone layer, and contain mycelium (while not as easily identified) at this stage. The aleurone cells lack large starch granules and are characterized by several spherical aleurone grains (Fig. 1D).

Longitudinal sections of the embryo reveal apparently healthy parenchyma cells 3 days after inoculation (Fig. 2A). The coleorhiza is composed of parenchyma cells that are bounded by inner and outer epidermal layers (Fig. 2B). The cell walls in the tip are slightly thicker-walled

than elsewhere in the coleorhiza (Bradbury *et al.*, 1956). The root cap is located at the tip of the radicle, and is easily recognized by the rows of cells composing it (Fig. 2C). Cells within the radicle differ from cells in the root cap; cells stack vertically, and are rectangular and pronounced in shape. The coleoptile lies next to the radicle, and meets in a v-shape backward turning curve (Fig. 2C). The scutellum stores food for the elongating embryo and at the time of germination becomes a digesting and absorbing organ that transfers food from the adjacent endosperm to the growing parts of the embryo (Bradbury *et al.*, 1956). The scutellum consists of parenchyma cells which vary considerably in size (Fig. 2D). The epithelium is located near the scutellum and is a layer of secreting cells that are highly saturated with blue staining dye (Fig. 2D).

Following eight days of disease development, both the embryo and endosperm are significantly altered due to infection by *P. semeniperda*. The endosperm begins to appear hollow and lacks endosperm cells within the center of the seed (Fig. 3A). The starch within some cells is partially consumed by mycelium, leaving behind remnants of the cell wall and causing cells to collapse and gradually withdraw toward the aleurone layer (Fig. 3B). Mycelium lines the testa and is evident within both large starch-containing endosperm cells and aleurone cells. While aleurone grains are still visible, some aleurone cells appear partially dissolved (Fig. 3C), resulting in locations that lack aleurone cells (Fig. 3D).

Within the embryo, mycelium is evident on the outside layer of the testa (Fig. 4A). The cell walls within the coleoptile appear less defined. Mycelium is evident throughout the entire embryo (Fig. 4B). Cells within and surrounding the radicle are less rectangular and defined. Cell walls within the radicle lack prominent rectangular shape and contain large quantities of mycelium (Fig. 4C). The scutellum also contains large amounts of mycelium. The epithelium cells appear to have collapsed, causing a large space to have been created between the scutellum

and epithelium (Fig. 4D). While many cells within the embryo contain mycelium, the integrity of cells is still evident, unlike cells within the endosperm. Embryonic cells contain very little starch (Bradbury *et al.*, 1956) and are living, unlike endosperm cells.

Following fourteen days of disease development, *P. semeniperda* has successfully caused severe damage to both the endosperm and embryo. The development of fungal stromata is apparent on the outside of the caryopsis (Fig. 5A). Stromata appear to develop within the testa, rather than from the endosperm; mycelium expands, eventually leading to stromata eruption toward the surface of the seed. Mycelium is evident within the center of the cell-vacant endosperm (Fig. 5B). Aleurone cells are less prominent, while cells on the outer edges of the endosperm still contain starch. Mycelium is evident within the testa (Fig. 5C) and consumes the starch within endosperm cells (Fig. 5D).

When comparing disease development within the embryo to seeds infected for only 8 days, the cells appear less prominent (Fig. 6A). The radicle end closest to the rootcap is collapsed, while mycelium is apparent all throughout the embryo (Fig. 6B). Cells within the radicle lack definition and some are altogether consumed. Additionally, cells to the right and left of the radicle contain significant quantities of mycelium (Fig. 6C). The epithelial layer near the scutellum is significantly collapsed; the endosperm gives way to the epithelial cells as it falls (Fig. 6D). In comparison to the endosperm, the embryo is more resistant to onset of disease development, but is ultimately impaired by the fungus.

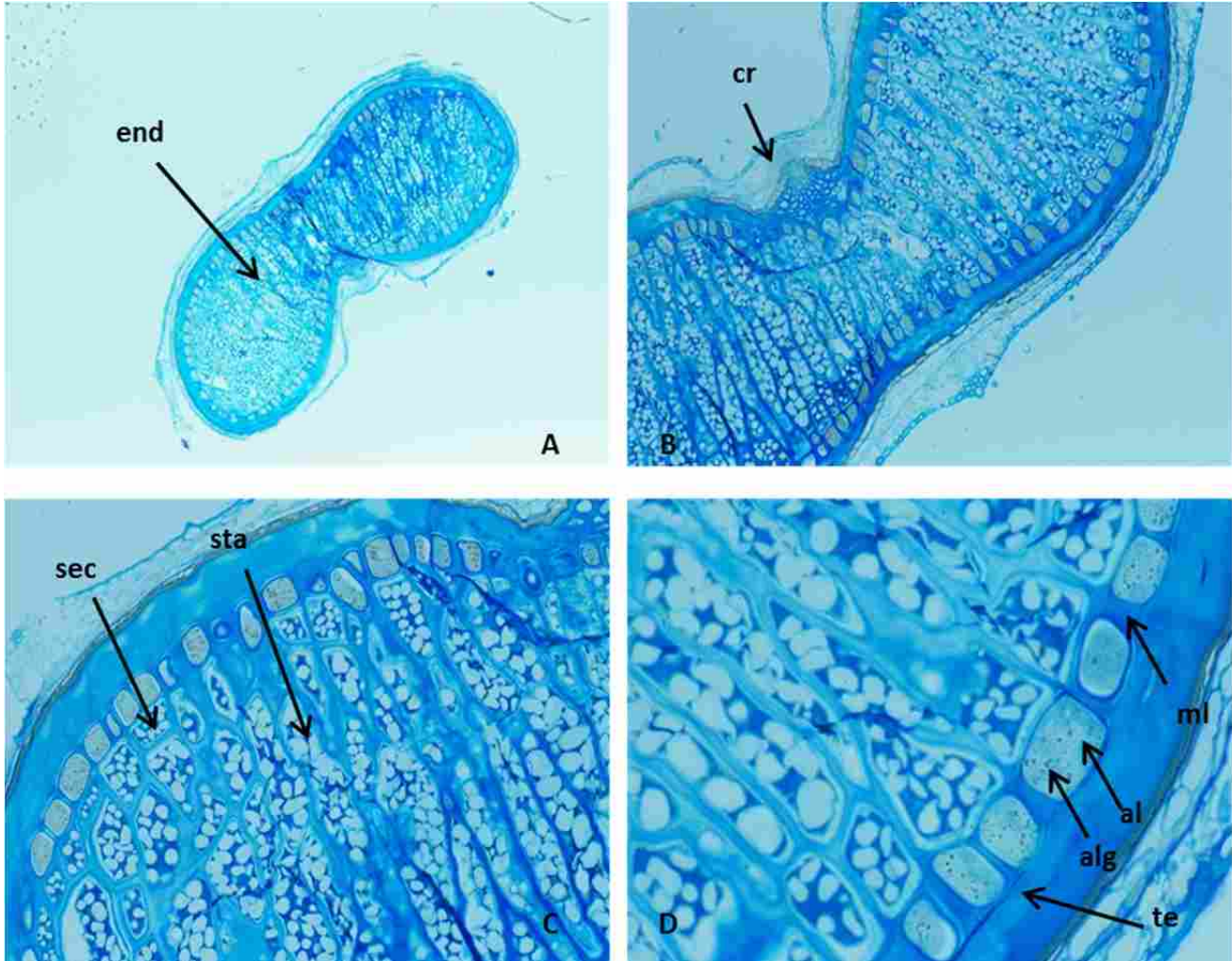
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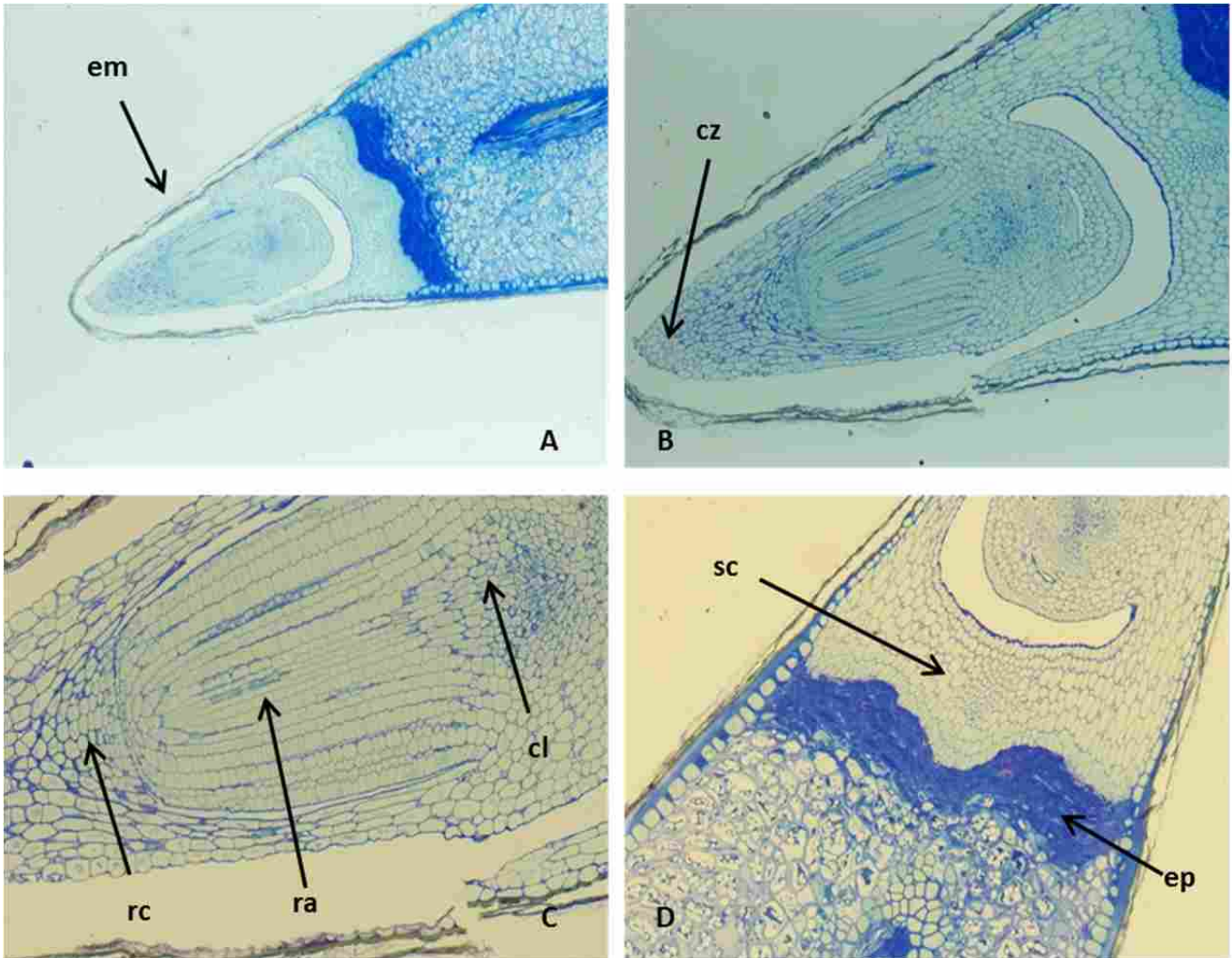


## FIGURES



**Figure 1. Disease Development of *P. semeniperda* Within the Endosperm in Three Days.**

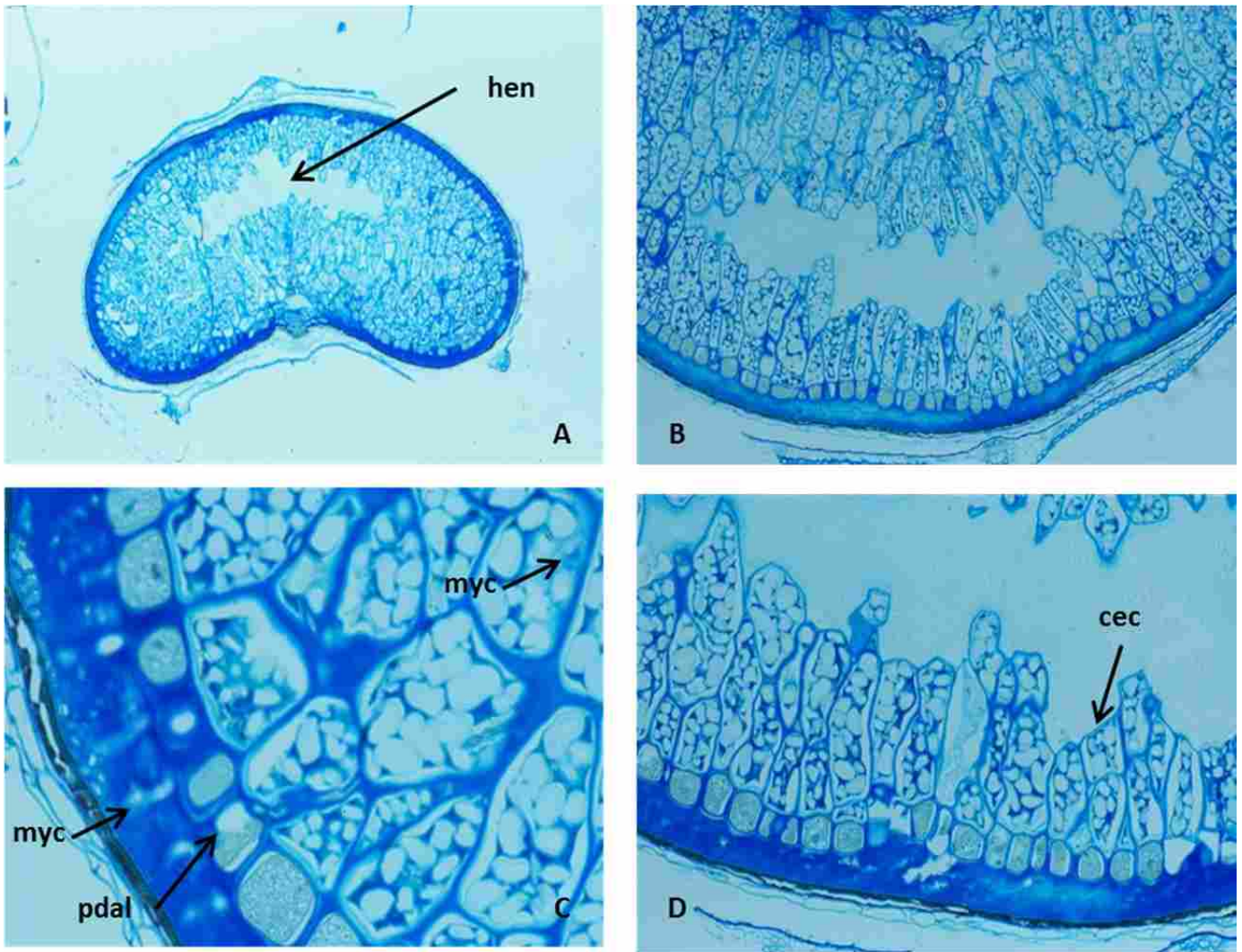
The endosperm of a dormant *Bromus tectorum* seed was cross-sectioned following three days of disease development and viewed with a light microscope. Images are taken of the following: (A) the endosperm (end), (B) higher magnification of the endosperm and crease (cr), (C) small (sec) and large endosperm cells containing starch granules (sta), and (D) the aleurone layer, containing individual aleurone cells (al) and aleurone granules (alg), separated by the middle lamella (ml) below the testa (te).



**Figure 2. Disease Development of *P. semeniperda* Within the Embryo in Three Days.**

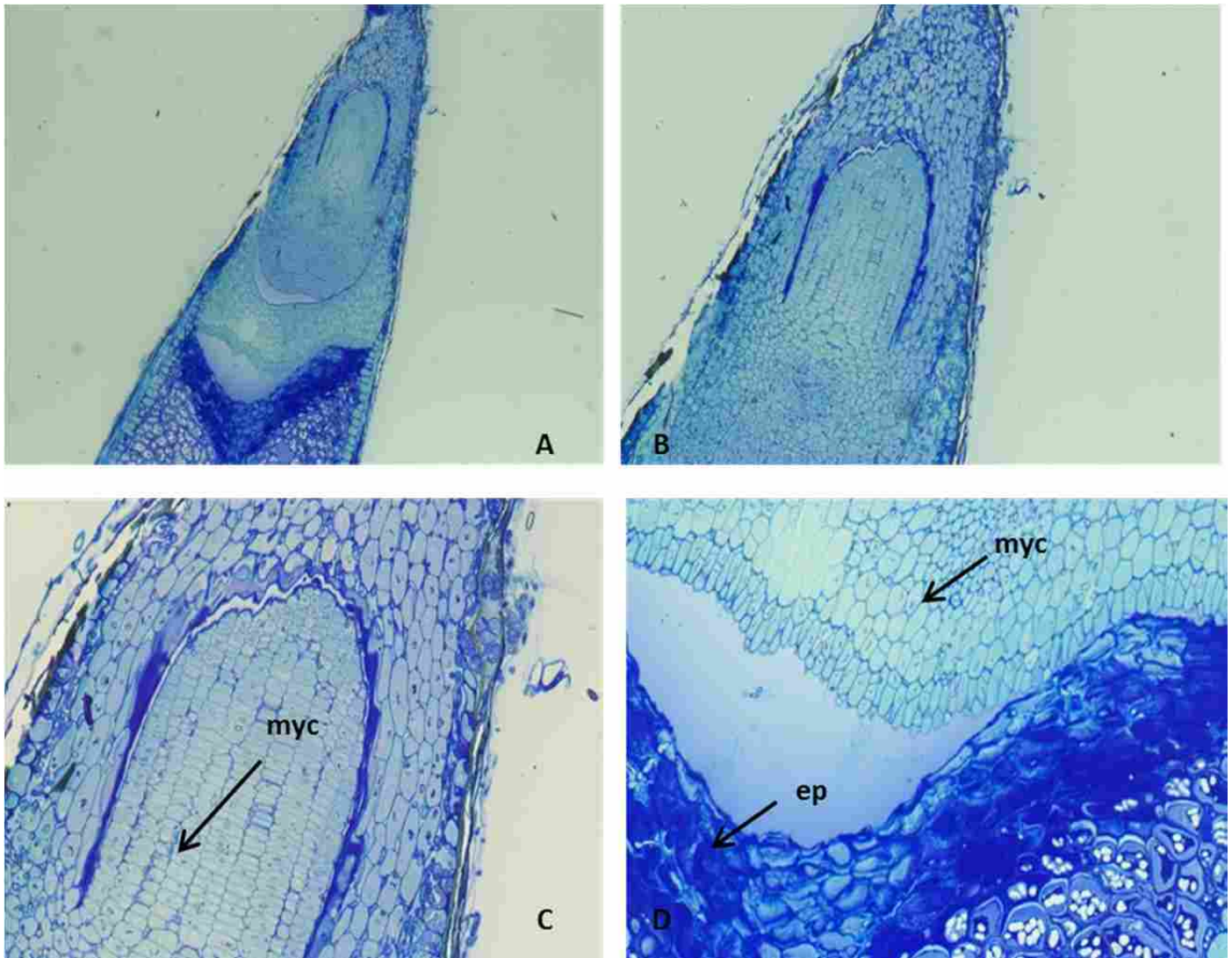
The embryo of a dormant *Bromus tectorum* seed was longitudinally-sectioned following three days of disease development and viewed with a light microscope. Images are taken of the following: (A) the embryo (em), (B) the coleorhiza (cz), (C) the rootcap (rc), radicle (ra), and coleoptile (cl), and (D) the scutellum (sc) above the epithelium (ep).





**Figure 3. Disease Development of *P. semeniperda* Within the Endosperm in Eight Days.**

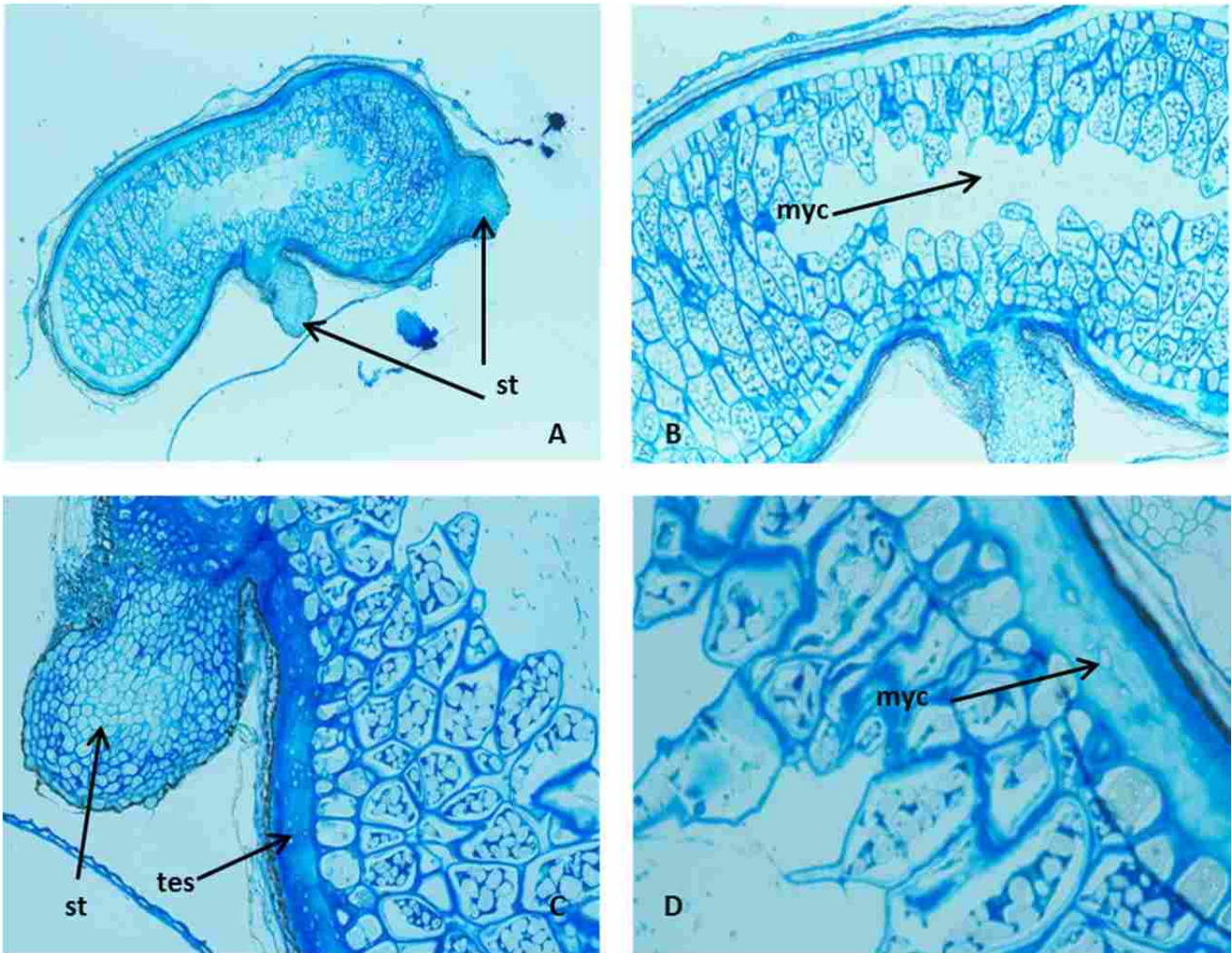
The endosperm of a dormant *Bromus tectorum* seed was cross-sectioned following eight days of disease development and viewed with a light microscope. Images are taken of the following: (A) the hollow endosperm (hen) that has been consumed by mycelium and correlates with SEM images of fractured infected seeds following eight days (Finch, 2013), (B) a higher magnification of the endosperm, (C) mycelium (myc) infected endosperm cells and a partially dissolved aleurone cell (pdal), and (D) a partially consumed endosperm cell (cec).



**Figure 4. Disease Development of *P. semeniperda* Within the Embryo in Eight Days.**

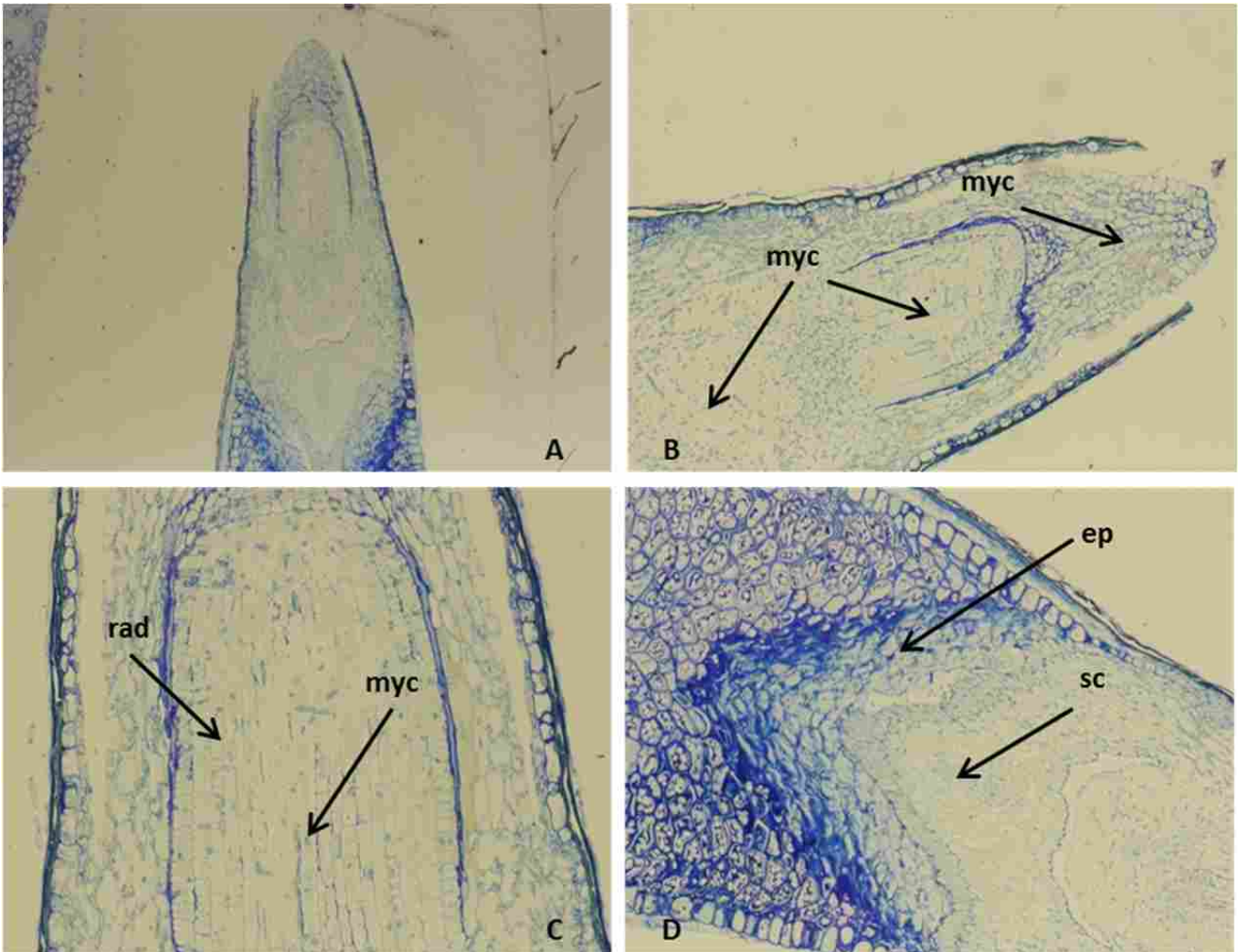
The embryo of a dormant *Bromus tectorum* seed was longitudinal-sectioned following eight days of disease development and viewed with a light microscope. Images are taken of the following: (A) the embryo, (B) a higher magnification of the embryo, (C) mycelium (myc) infected cells within the radicle, and (D) a collapsed epithelium (ep) below mycelium (myc) filled cells in the scutellum.





**Figure 5. Disease Development of *P. semeniperda* Within the Endosperm in Fourteen Days.**

The endosperm of a dormant *Bromus tectorum* seed was cross-sectioned following 14 days of disease development and viewed with a light microscope. Images are taken of the following: (A) the endosperm with stromata (st) protruding from the caryopsis, (B) a higher magnification of the endosperm containing mycelium (my), (C) the testa (tes) containing mycelium and the development of a stroma (st), and (D) consumed endosperm cells surrounded by the testa containing mycelium (myc).



**Figure 6. Disease Development of *P. semeniperda* Within the Embryo in Fourteen Days.**

The embryo of a dormant *Bromus tectorum* seed was longitudinally-sectioned following 14 days of disease development and viewed with a light microscope. Images are taken of the following: (A) the embryo, (B) a higher magnification of the endosperm containing large quantities of mycelium (my), (C) collapsed cells within the radicle (rad) containing mycelium (myc), and (D) the scutellum (sc) next to a collapsed epithelium (ep) that is withdrawing toward the endosperm.

## **APPENDIX B: ADDITIONAL METHODS**

Scanning Electron Microscopy and Cross-sectioning of the Seed Embryo and Endosperm

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## **CROSS SECTIONING- EMBRYO AND ENDOSPERM**

### **Inoculation (Embryo and Endosperm)**

Seeds of *B. tectorum* L. were collected from a wild population at the Brigham Young University Research Farm (Spanish Fork, Utah, USA) in June 2011. Seeds were cleaned by hand and stored in a -10°C freezer to maintain dormancy. *Pyrenophora semeniperda* inoculum originated as a moderately virulent strain collected from Whiterocks, Utah, USA, and was produced as described by Meyer *et al.* (2010). Seeds were placed in a vial of approximately 3 mg of spores and 1 ml of distilled water, and shaken for 10 seconds. Inoculated seeds were removed from the vial and placed on top of two blue germination blotters (Anchor Paper, St. Paul, Minnesota, USA) inside a Petri dish within plastic sleeves and incubated with cycles of 12 h fluorescent light/12 h dark at constant 20°C, which is near optimum for the pathogen (Campbell *et al.*, 1995).

*NOTE:* DO NOT use ANY form or mixture of talc when inoculating seeds for sectioning. This ruins your diamond knife.

### **Fracturing (Embryo and Endosperm)**

At days 3, 8, and 14, infected seeds were removed from Petri dishes and fractured (Figure 1, dotted line) by placing them in liquid nitrogen and dissecting them under a light microscope with a medical blade. Single cross sections were taken (Figure 3). Seeds were scarified on the opposite side of the embryo to allow the Spurr's resin to better infiltrate into the sample (Figure 1). Fractured portions of the seed were placed in vials of primary fixative (2% glutaraldehyde solution buffered with sodium cacodylate at pH 7.3) for 2-7 days.



## **Fixation**

After fixation, seeds were washed with cacodylate buffer. Contents of the vial were replaced with 2 ml of cacodylate buffer six times for 10 minute increments. Afterward seeds were secondarily fixed and stained with 1% osmium (OsO<sub>4</sub>) solution buffered with sodium cacodylate at pH 7.3. Following secondary fixation, the osmium was replaced with distilled water and seeds were washed six times for 10 minute increments. After the samples were washed with distilled water, they were dehydrated with a graded series of acetone (10, 30, 50, 70, 95%). Each concentration of acetone within the graded series lasted 10 minutes. Following the 95% concentration, seeds were dehydrated with 100% acetone, three times for 10 minute increments.

Occasionally, samples were left in 70% acetone overnight, and completely dehydrated the following day.

## **Spurr's Resin (Spurr, 1969)**

The low-viscosity embedding medium for fixed samples was composed of: ERL-4221, 5 g, D.E.R. 736 (diglycidyl ether of polypropylene glycol) 3.5 g, NSA (nonenyl succinic anhydride) 13 g, and D.M.A.E. (dimethylaminoethanol) 0.2 g. The medium was prepared by dispensing the components, in turn by weight, into a single flask. Using a rod, ingredients were stirred until the medium turned a honey-yellow color (about 3 minutes of stirring). (This is a low viscosity kit purchased from PELCO).

Following dehydration, fixed samples were placed in vials with a ratio of  $\frac{2}{3}$  100% acetone and  $\frac{1}{3}$  Spurr's resin. The vial was placed on a rocking tray machine for about 2 hours. Following the two hours, the contents of the vial (excluding the samples) were removed and

replaced with a ratio of ( $\frac{1}{3}$ ) 100% acetone and  $\frac{2}{3}$  Spurr's resin. Again the vial was placed on a rocking tray machine for 2 hours. Following the two hours, the liquid contents of the vial were replaced with 100% Spurr's resin. The vial was left on the rocking tray for an additional two hours. Next, samples were removed and placed in a flat plastic bottle cap containing Spurr's resin. The caps were then placed in a 70°C oven overnight to allow the resin to polymerize (about 12 hours or longer).

Following polymerization, samples were removed from the oven. The plastic bottle cap was removed, and a round, firm plastic resin disk remained. Samples were cut out of the plastic resin disk with a jeweler's saw and mounted on resin stubs with cyanoacrylate glue (super glue). Samples were again placed in a 70°C oven for 10-15 minutes to allow the glue to set. Once set, samples were trimmed with razor blades in the shape of a trapezoid prism for cut by a microtome (Bozzola and Russell, 1992, pg 66-70).

Once trimmed, samples were placed in a microtome and cut with an 8mm diamond knife (Figure 3, represented by dotted lines). Samples were cut in 1µm-thick sections (Figure 3).

### **Staining Protocol**

The standard blue stain used for dyeing slides was made by weighing 0.25 grams of Borax, Toluidine Blue, and Azure II in a glass vial, followed by 25 ml of distilled water. The mixture was stirred on a hotplate at low heat for ten minutes with a magnetic stir bar. Before use, the mixture was warm to touch (~62°C).

Using a syringe, liquid was drawn and the needle was removed and replaced with a syringe filter (Thermo Scientific Nalgene Syringe Filter, 0.2µm). Dye was applied, fully

covering slides. Slides sat with the dye for 5-15 seconds (without heat), then rinsed with distilled water and placed on a hot plate to dry, or allowed to dry on their own (30-60 minutes).

Following the staining process, cover slips were placed on slides. To do so, completely dry slides were immersed in Zylene for 10-15 seconds. Using permount sealant, cover slips were adhered to slides carefully, allowing no air bubbles to form. Weights were placed on top of cover slips and allowed to sit for at least 48 hours. Slides were later cleaned for better visibility with Zylene-immersed Q-tips.

## **SEM PREPARATION**

### **Single-Spore Inoculation**

Seeds of *B. tectorum* L. were collected from a wild population at the Brigham Young University Research Farm (Spanish Fork, Utah, USA) in June 2011. Seeds were cleaned by hand and stored in a -10°C freezer to maintain seeds in the dormant condition. The *P. semeniperda* inoculum originated as a moderately virulent strain collected from Whiterocks, Utah, USA, and was produced as described by Meyer *et al.* (2010). To perform single-spore inoculation, *P. semeniperda* spores were placed on a polycarbonate membrane and hydrated for 20 minutes with distilled water. Using the end of a 2 mm diameter glass probe (made into a needle using flame), individual spores were picked up with the needle under a light microscope and placed on a seed. Inoculated seeds were then placed on top of two blue germination blotters (Anchor Paper, St. Paul, Minnesota, USA) and a polycarbonate membrane inside a Petri dish within plastic sleeves with cycles of 12 h fluorescent light/12 h dark at constant 20°C, which is near optimum for the pathogen (Campbell *et al.*, 1995).

*NOTE:* Polycarbonate membrane was used to prevent germination blotter fibers from contaminating the samples and ruining the quality of the images obtained by the scanning electron microscope.

Additional images were taken of dormant *B. tectorum* seeds that were subjected to one of the following treatments prior to inoculation: 1) removal of the lemma and palea (naked caryopsis), 2) autoclaved (dead) and dried for 24 hours, 3) surface sterilized (for either a short or long period, specifically by immersing seeds for one minute in ethyl alcohol (70%) and one minute in bleach (10%), or two minutes in ethyl alcohol and two minutes in bleach), 4) removal of the lemma and palea, followed by either short or long surface sterilization as described, and 5) removal of the lemma and palea followed by autoclaving. Surface sterilized seeds were rinsed with sterile distilled water before seeds were inoculated.

### **Fixation**

Seeds infected for 1-21 days were placed in a vial of primary fixative (2% glutaraldehyde solution buffered with sodium cacodylate at pH 7.3) for 2-7 days. After fixation, seeds were washed with cacodylate buffer. Contents of the vial were replaced with 2 ml of cacodylate buffer six times for 10 minute increments. Following washing, seeds were secondarily fixed and stained with 1% osmium tetroxide (OsO<sub>4</sub>) solution buffered with sodium cacodylate at pH 7.3. Following secondary fixation, the osmium was replaced with distilled water and seeds were washed six times for 10 minute increments. After the samples were washed with distilled water, they were dehydrated with a graded series of acetone (10, 30, 50, 70, 95%). Each series lasted 10 minutes. Seeds were then dehydrated with 100% acetone, three times for 10 minute increments. Following dehydration, seeds were critical point dried using a CPD machine.

### **Mounting of Samples**

In order to mount samples, conductive carbon-based tape was attached to the top of an aluminum mount, 1/2 inch in diameter. Samples were placed on the tape.

### **Coating of Samples**

Mounted samples were placed in a sputter coater machine. With standard settings, the machine applied about 15 nm of a gold/palladium layer.

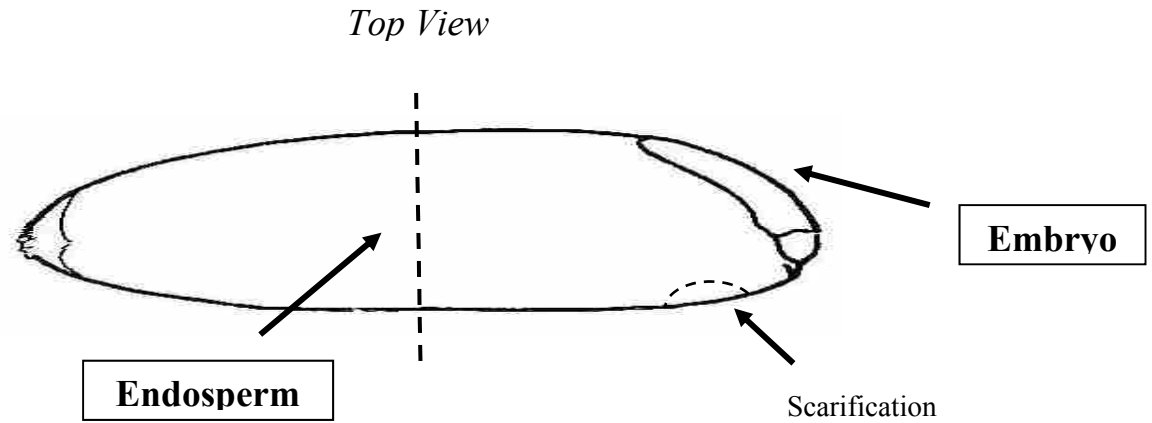
### **Freeze Dryer**

To obtain images of un-germinated and germinated spores at specified times (6, 8 hours), freeze drying was used. We found that we obtained more realistic/better images due to the sensitive nature of the freeze dryer. The following process (times and temperatures) were used to freeze dry spores. Ten segments were used (Table 1). Dry samples were mounted on an aluminum mount, as explained in the “Mounting of Samples” section.

## REFERENCES

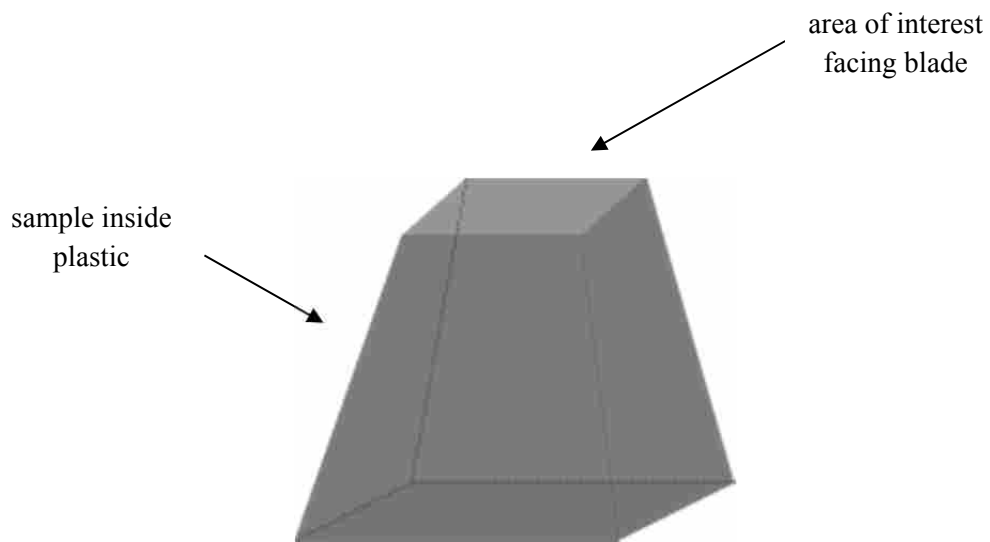
- Bozzola, John J. and Russell, Lonnie D.** (1992) “Electron Microscopy: Principles and Techniques for Biologists”, *Jones and Bartlett Publishers, Boston, MA*. ISBN: 0-86720-126-6.
- Campbell, M.A., Medd, R.W. and Brown, J.F.** (1995) Growth and sporulation of *Pyrenophora semeniperda* in vitro: effects of culture media, temperature and pH. *Mycological Research* **100**, 311–317.
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## FIGURES



**Figure 1. The Top View of a *Bromus tectorum* Seed.**

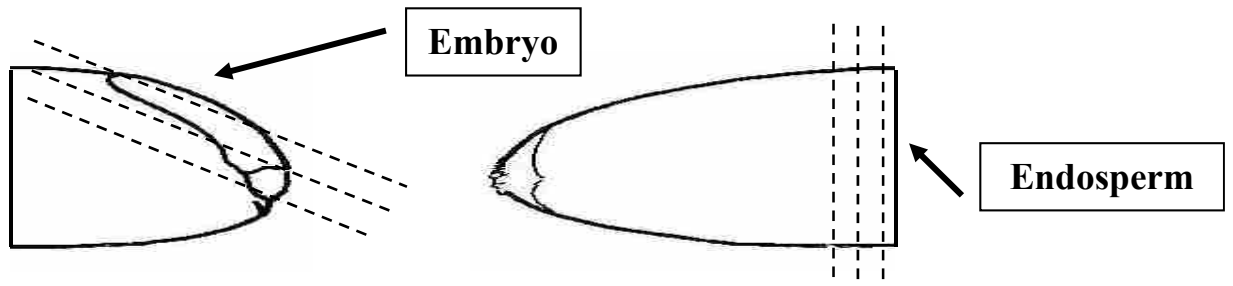
A dormant *B. tectorum* seed was fractured at the dotted line in order to obtain smaller samples to be cross sectioned for view of the embryo and endosperm. Small sections of the seed were scarified to better permit the resin to infiltrate into the seed.



**Figure 2. Plastic Trapezoid Containing Sample of Interest.**

Resin capsules containing samples of interest were cut in the shape of a trapezoid. The area of interest was faced toward the blade for cross sectioning.





**Figure 3. Cross Sectioning Angles.**

Cross sections, as represented by the dotted lines, were taken of both the embryo and endosperm. Cross sectioned measured 1 $\mu$ m-thick sections, approximately.

## TABLES

**Table 1. Freeze Drier Segments, Times, and Temperatures.**

Samples were freeze dried according to the segments, times, and temperatures listed in the above table. These segments provided the best outcomes.

<u><i>Segment</i></u>	<u><i>Time</i></u>	<u><i>Temperature</i></u>
1	5-10 minutes	-120 <sup>0</sup> C
2	2 hours	-120 <sup>0</sup> C
3	30 minutes	-100 <sup>0</sup> C
4	2 hours	-100 <sup>0</sup> C
5	1 hour	-80 <sup>0</sup> C
6	4 hours	-80 <sup>0</sup> C
7	1 hour	-50 <sup>0</sup> C
8	4 hours	-50 <sup>0</sup> C
9	2 hours	0 <sup>0</sup> C
10	2 hours	25 <sup>0</sup> C