

Project Title: Enhancing the Effectiveness of Annual Grass Weed Biocontrol with the Black Fingers of Death Pathogen (*Pyrenophora semeniperda*) on Intermountain Rangelands

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I. Abstract

A major problem in post-fire restoration of semi-arid shrublands dominated by annual bromes is the presence of carryover seed banks that cannot be controlled using conventional methods. Plants from these carryover seeds can potentially provide significant competition for seeded species in the years immediately following treatment, even if the current year stand of annual grass weeds was effectively controlled at the time of seeding. In this project we extended our previous investigations on the feasibility of using a naturally occurring seed pathogen, the ascomycete *Pyrenophora semeniperda*, as a biocontrol organism for eliminating this carryover seed bank. Our study had three components. In the first component we extended our evaluation of field-applied pathogen inoculum in the context of a native grass seeding. We found that, while

there was no conclusive evidence that the level of control we achieved had a positive effect on seeding success, there was no negative impact of the inoculum addition treatment on the seeded grass, in contrast to the strong negative impact of imazapic at one site. In some cases the inoculum addition effectively controlled the carryover seed bank and in one case it significantly reduced cheatgrass density and biomass the second year after treatment, showing the potential for decreasing competition in the first few years after seeding. In the second study component, we carried out detailed investigations of variation among pathogen strains. We determined that the wide range of mycelial growth rates observed in pathogen populations was the result of temporally varying selection on strains with different growth rates, with slow growing strains more successful on nondormant seeds in the autumn seed bank and fast growing strains more successful on dormant seeds. This suggests that mixtures of strains with contrasting traits might provide both elimination of the carryover seed bank of dormant seeds and quick knock down of germinating seeds in the seed bank, an idea that is currently under investigation. We also initiated a study of breeding methodologies to create pathogen strains with novel traits outside the natural range of variation that could be more useful in biocontrol. In the third study component, we determined in cross-inoculation studies that there is a complete lack of host specialization in this pathogen on a suite of weedy annual grasses, including several annual bromes as well as medusahead. Strains from each pathogen population varied widely in aggressiveness, but aggressive strains were more aggressive on both cheatgrass and the host of origin. This indicates that it will not be necessary to select host-specific strains in order to develop a biocontrol technology that will be effective across a wide range of problematic annual grass weeds. In summary, our study provided a wealth of new information on this pathogen and its potential as a biocontrol organism for annual grass weed biocontrol. The remaining obstacle to its widespread use is the need for development of an economically feasible inoculum delivery system that can deliver sufficient quantities of a consistently potent product that yields a high level of control even in the face of environmental variation and known variation in general resistance to pathogen attack in host populations. Selection or breeding of strains that are more efficacious for biocontrol will also facilitate the achievement of this goal.

II. Background and Purpose

Annual bromes on western rangelands are a major contributing cause of wildfires that have immense economic and environmental costs. Black fingers of death (*Pyrenophora semeniperda*) is a naturally occurring ascomycete fungal pathogen that is known to kill large numbers of seeds in annual brome seed banks. Our ultimate goal is to develop this organism for biocontrol of annual bromes and possibly other annual grass weeds through: (1) complete eradication of the dormant persistent seed bank, and (2) quick knock-down of a high proportion of germinable (non-dormant) seeds. The purpose of this biocontrol is to function as an inundative mycoherbicide agent to temporarily reduce annual brome competition in the context of restoration seeding, particularly post-burn seeding. This is in contrast to the goal of long-term weed reduction directly through the impact of the pathogen (the model for classical biocontrol). For mycoherbicide biocontrol, the effect of the treatment itself is transient, but the potential positive effect on rangeland health persists through increased seeding success and establishment of native perennial plant communities that are resistant to annual brome reinvasion.

With previous funding from the Joint Fire Science Program (2007-2010), we produced evidence that this pathogen has potential for biocontrol of annual brome dormant carryover seed

banks, and that a sizeable fraction of potentially germinable (non-dormant) seeds can be killed as well. The upper threshold for annual brome seed bank density for successful establishment of natives is estimated at 300 viable seeds per m². Our best treatments have resulted in viable seed densities well below this threshold, and in some cases even below the limit of sensitivity of our sampling methodology (17 viable seeds per m²). These treatments have included the use of herbicides to eliminate the seedlings that resulted from germination of non-dormant seeds, but these herbicides alone can do nothing to eliminate the bank of ungerminated seeds. The herbicides prevent seed set by eliminating germinated seeds or established plants, and the pathogen destroys the persistent bank of ungerminated seeds. At this stage, it is necessary to use both herbicide and biocontrol treatments to achieve complete or near-complete control.

In conducting the research funded under the Joint Fire Science project reported upon here, we focused on three primary study objectives:

- 1) Determine effectiveness of the pathogen for persistent annual brome seed bank biocontrol in terms of positive and potentially negative impacts on seeded natives under realistic restoration scenarios.
- 2) Determine the potential for increasing effectiveness of annual brome seed bank biocontrol with this pathogen by evaluating among-strain variation in a suite of potentially important traits and their effect on biocontrol efficacy.
- 3) Examine the potential for biocontrol effectiveness on other important annual grass weeds using laboratory cross-inoculation trials.

III. Study Design and Methodology

Objective 1. We used field experiments with split plot designs at two sites across three years to examine the effect of pathogen inoculum addition on killed and viable seed densities in the cheatgrass carryover seed bank, cheatgrass tiller (plant) density and biomass production for two years after application, and emergence, establishment and survival of a desirable native grass, squirreltail (*Elymus elymoides*). Each experiment included 10 block replicates. The main plots were 3 x 5 ft, large enough to accommodate both four one-square-foot precision seeding plots for planting squirreltail seeds and areas for evaluation of cheatgrass carryover seed banks, tiller density, and biomass production over two years. For each subplot, 20 squirreltail seeds mounted on toothpicks for ease of seedling detection were planted in a grid at 5 cm spacing.

Pathogen inoculum was added to the main plots on a vermiculite carrier at a rate of 15 g/square foot. Supplemental water was added as 25mm at planting and 25 mm one week later, while the fungicide treatment (captan) was applied as a slurry to the seeds on their toothpicks and allowed to dry. Experiments were planted in September each year, while squirreltail emergence and survival were evaluated in November, April and May of the first year and again in May of the second year. For seed bank evaluation, three 6 cm diameter soil cores were obtained from the designated area of each main plot the first spring and the second spring after planting. Seed bank samples were sorted by hand to remove cheatgrass seeds and score them as either viable (germinable) or killed by the pathogen (visible stromata protruding). Tiller counts were made from a circular area 6 inches in diameter each spring, while aboveground biomass was obtained

from a square-foot area that included the tiller count area. Separate areas within each main plot were sampled each spring.

For the first experiment, installed at each site in fall 2012, we combined the pathogen inoculum addition treatment with an imazapic treatment to eliminate the current-year established cheatgrass stand, resulting in a factorial design with four combinations and 40 main plots. Each main plot included four squirreltail seeding subplots with supplemental water and fungicide treatment combined factorially for a total of 160 subplots.

For the second experiment, installed at each site in fall 2013, we first greatly reduced the current year cheatgrass stand by applying glyphosate to prevent seed set in spring 2013 over the entire area. The three main plot treatments were pathogen inoculum applied a year in advance (fall 2012), pathogen inoculum added at the time of planting (fall 2013) and a no inoculum control, for a total of 30 main plots. There was no supplemental water treatment; each main plot included two subplots with fungicide and two subplots without fungicide, for a total of 120 subplots.

Data were analyzed using SAS Proc Mixed models appropriate to each experimental design.

Objective 2. Detailed methods for the strain trials can be found in Finch et al. (2013), Barth et al. (2015), and Meyer et al. (2015). We applied these methods to 10-12 strains selected for a range of mycelial growth rates in the screening experiments described in the results, with four replicates of 25 or 50 seeds for each treatment combination. Seeds from the Spanish Fork Farm UT cheatgrass population were used in most tests in an effort to hold host genetics constant. Data were analyzed using analysis of variance or linear regression appropriate to the experimental designs.

Objective 3. Detailed methods for the cross-inoculation experiments with pathogen strains from multiple weedy annual grass hosts can be found in Beckstead et al. (2016). Briefly, we obtained pathogen strains and seeds from *Bromus rubens*, *Bromus diandrus*, *Bromus arvensis* (formerly *Bromus japonicus*) and *Taeniatherum caput-medusae* as well as from *Bromus tectorum*. We genetically characterized these strains using ITS and microsatellite (SSR) markers to determine if there was genetic differentiation among strains on different hosts. We performed cross-inoculations with six strains from cheatgrass and each alternate host on dormant seeds of each host at low inoculum loads to determine whether host specialization occurs on different annual grass hosts, i.e., whether strains would be most aggressive and cause highest mortality on seeds of their host of origin. We also tested the hypothesis that host seeds would be highly susceptible to infection by their own strains whether dormant or nondormant, but would be more tolerant to infection when nondormant and able to escape through germination. Data were analyzed using SAS Proc Mixed for the appropriate experimental designs.

IV. Key Findings

Objective One – Pathogen Effectiveness for Persistent Seed Bank Biocontrol under Realistic Restoration Scenarios

We carried out two full sets of two-year field inoculations as described above at each of two cheatgrass monoculture sites, Coonskin Butte near Twin Falls, Idaho, and Haven Flats on the Hanford Reach National Monument in Washington.

In the first experiment at Coonskin Butte, there was no autumn emergence of squirreltail without supplemental water, and even in the watered plots emergence was only 21%. Spring emergence in the unwatered plots was also low, and only 5% of seeds planted were present as seedlings overall in April 2013. There were no significant effects of pathogen inoculum addition, imazapic, or fungicide treatment on emergence or first year survival. At the end of the second year (2014), 1.7% of the planted squirreltail seeds were present as established plants. There was a slight but significant trend for higher surviving plant numbers in the imazapic treatment (2.5% vs. 1.0% of planted seeds). No other treatment was significant.

At Haven Flats, autumn emergence averaged 64% and was not significantly affected by pathogen inoculum, imazapic, or watering treatments. The fungicide treatment increased emergence from 61% to 70%. There was no fungicide by pathogen inoculum interaction, indicating that the fungicide protected the seeds from some other pathogen already in the soil rather than from added pathogen inoculum. There was no effect of pathogen inoculum addition, watering, or fungicide addition on survival through the first spring, which averaged 27% of seeds planted. In contrast, the imazapic treatment had a major negative effect on squirreltail seedling survival, which was reduced to 3.6% of planted seeds. In the plots not treated with imazapic, survival through the first spring averaged 51% of planted seeds. The second year at Haven Flats was extremely dry, and no squirreltail seedlings remained alive at census in May 2014.

At Coonskin Butte, adding pathogen inoculum in the first experiment significantly increased disease levels in the carryover seed bank the first year but did not reduce viable seed densities below the threshold for control (300 viable seeds per m²). In the second spring, the pathogen inoculum treatment applied the previous year continued to have some effect, reducing viable seed density by half in the no-imazapic treatment. Imazapic had no effect on seed carryover the first year, but in the second year, this treatment had the lowest viable seed densities, well below the threshold for control, due to the effectiveness of the herbicide in preventing cheatgrass emergence, growth, and seed production in the first year.

At Haven Flats, adding pathogen inoculum in the first experiment also increased disease levels, the first year, though not as much as at Coonskin Butte, likely because disease levels were already high at Haven Flats. Viable seed density was low across all treatment the first year. There was no significant effect of pathogen inoculum on either disease levels or viable seed density the second year. As at Coonskin Butte, viable seed densities were significantly lower in the main plots that had received imazapic the previous year.

In the first experiment, imazapic reduced tiller number and biomass production of cheatgrass the first year to near zero at both sites. There was no effect of pathogen inoculum the

first year on either tiller number or biomass production at either site. In the second year, pathogen inoculum addition on the non-imazapic main plots at Coonskin reduced both tiller number and biomass production significantly relative to the control, reducing tiller number by half and biomass by one third, indicating that this pathogen does have the potential to reduce competition from cheatgrass the year following application under some circumstances. Tiller number and biomass production were even lower in the imazapic main plots, averaging about one third of the tiller number and one sixth of the biomass observed in the control main plots. At Haven Flats, cheatgrass experienced complete stand failure across all treatments due to drought conditions the second year, so no treatment effects were noted.

The second experiment was initiated in fall 2013 at each site. At Coonskin Butte, sufficient autumn precipitation in 2013 resulted in 42% squirreltail emergence, with no treatment effect due to pathogen inoculum treatment. Total emergence increased to 47% of seeds planted by April 2014. The fungicide treatment increased total emergence from 44 to 50%, but again there was no interaction with the pathogen inoculum treatment, indicating that the fungicide was offering protection from some other pathogen. Survival at the end of May 2014 averaged 33% of seeds planted, with no effect of pathogen inoculum treatment. A year later, in May 2015, survival averaged 4.2% of seeds planted or ca. 9% of seedlings emerging, with no treatment effects. At Haven Flats there was essentially no emergence of squirreltail seedlings due to the extreme drought conditions, and there were no surviving seedlings present in spring 2014 or spring 2015.

At Coonskin Butte there was no consistent effect of pathogen inoculum addition on disease levels in the seed bank or viable seed density in the second experiment, though there was a trend the second year for higher disease levels in the pathogen inoculum treatments than in the control. There were also no significant effects of pathogen inoculum treatment on either cheatgrass tiller number or biomass production, in contrast to the result from the first experiment. At Haven Flats the first year, pathogen inoculum addition significantly increased disease levels in the seed bank and decreased viable seed density, particularly when added the year prior to seeding. This treatment reduced viable seed density relative to the control by a factor of four (from 9.9 to 2.5 viable seeds per dm^2 , a viable seed density below the threshold for control). Because of cheatgrass stand failure in 2013-2014, the seed bank at Haven Flats contained very few seeds in spring 2015, and viable seed densities were below the threshold for control regardless of treatment. There was no cheatgrass production at Haven Flats in 2014, resulting in zero values with no treatment differences for tiller number and biomass. In 2015, the cheatgrass stand showed weak recovery from the seed bank, with no effects due to pathogen inoculum treatment.

We obtained conflicting results with the imazapic treatment. It effectively eliminated cheatgrass stand establishment and had no effect on squirreltail emergence. However, its effect on squirreltail survival varied by site. At Coonskin Butte the effect on survival was largely null or even slightly positive, whereas at Haven Flats, imazapic caused almost complete mortality of emerged squirreltail seedlings the first year. These results confirm those of other workers that imazapic can have unpredictable negative effects on native seedlings. This makes it a potentially poor substitute for biocontrol in spite of its effectiveness at preventing stand establishment of weedy annuals.

In summary, we were not able to detect a positive effect of pathogen inoculum addition on squirreltail seedling establishment and survival, but we also detected no negative effect on emergence and no protective effect of fungicide, indicating that pathogen inoculum addition did no harm to squirreltail seeds. Our failure to detect a positive effect on survival could have been due to experimental design limitations, as the absolute number of seedlings surviving in any treatment was generally small, making it hard to detect treatment effects. Larger-scale experiments under a range of different weather scenarios would be required to determine for certain whether this pathogen has biocontrol potential in terms of enhancing native grass establishment. Pathogen inoculum addition did reduce the carryover seed bank below the threshold for control in some cases, and in one case we detected a significant negative second-year effect of pathogen inoculum addition on cheatgrass tiller number and biomass production. Establishment failure of both squirreltail and cheatgrass at Haven Flats in 2013-2014 reduced our chances of detecting treatment effects, as an unavoidable consequence of an exceptionally dry year.

Objective Two – Among-strain Variation in Mycelial Growth Rate, Toxin Production, Environmental Tolerance, and Aggressiveness on Dormant vs. Nondormant Seeds

Our first set of experimental trials with a set of pathogen strains newly isolated in 2010 were designed to build on our earlier work exploring the relationship of mycelial growth rate to the ability of the pathogen to kill dormant vs. nondormant seeds. Our hypothesis was that fast-germinating strains would be better able to overcome resistance and cause mortality of dormant seeds at the low inoculum loads found in spring seed banks, whereas slow-germinating strains would be better able to kill non-dormant seeds at high loads in the autumn seed bank (Meyer et al. 2015). We also addressed the mechanism by which slow-growing strains could kill fast-germinating seeds. Our extensive work on secondary metabolites produced by the fungus revealed that the potent phytotoxin cytochalasin B was produced in massive amounts, along with lesser amounts of phytotoxins belonging to other classes of secondary metabolites (Masi et al. 2014a, b, c, d). Cytochalasin B was produced by the fungus only in solid culture on seeds or in liquid culture that contained seed constituents, showing that its production was induced by the presence of the host. Living cheatgrass seeds had a mechanism to at least partly suppress the production of cytochalasin B relative to production on pre-killed seeds, offering further evidence of its importance in pathogenesis. Finally, we showed that mycelial growth rate in this fungus was negatively correlated with cytochalasin B production, indicating that there is indeed a trade-off between mycelial growth and toxin production. This explains why slow-growing strains were better able to kill nondormant seeds, that is, because they produced more of the toxin that could cripple the seed and prevent escape through germination. In contrast, we showed that faster-growing strains of the fungus were better able to kill dormant seeds at low inoculum loads. The maintenance of high variation in mycelial growth rate in populations of this pathogen was hypothesized to be related to temporally varying selection on strains with different growth rates, with slow strains favored in the autumn seed bank and fast strains favored on dormant seeds in the spring.

We also performed a large set of experiments on response of the pathogen to environmental conditions, specifically temperature and water potential. We first demonstrated that the pathogen could germinate and grow over a wide range of temperatures, but that temperatures typical of autumn and spring seed banks were optimal. We also determined that the

pathogen could germinate and grow at water potentials far below those that would allow germination of host seeds or seedling growth, giving it an advantage under water stress conditions in the field (Finch et al. 2013). In order to model these processes under field conditions, we developed hydrothermal time models for pathogen conidial germination and mycelial growth that were in parallel to models developed earlier by our team to predict host seed germination in the field. This work represents the first time that hydrothermal time concepts traditionally applied to the prediction of seed behavior have been used to model the germination and growth of a fungus (Barth et al. 2015).

We tied the results of these basic hydrothermal time studies to patterns of seed mortality across temperature and water stress gradients, and also to the outcome in terms of seed mortality following intermittent hydration, a condition that would commonly occur in the field (Finch 2013, Hawkins 2014). We found that virtually all conditions that included fluctuating water potentials or water potentials suboptimal for germination tended to favor pathogen activity and increase host seed mortality. These results strongly suggest that the reason for higher levels of disease at drier sites with more sporadic fall moisture relative to more mesic sites is that time spent at water potentials that prevent seed germination but still permit pathogen activity can result in mortality of large numbers of nondormant seeds. The optimal water potential for mycelial growth rate was -1.5 MPa, which is near the lower limit for host seed germination and growth. Pathogen conidia could germinate at water potentials as low as -6 MPa as long as temperature was near the optimum (20C).

The experiments discussed above were carried out with a representative pathogen strain but did not address variation among strains in germination, growth rate, or ability to cause seed mortality as a function of temperature and water potential. In multiple-strain trials we generally found more variation among strains under suboptimal temperature conditions than at the optimum. For example 6-hr conidial germination percentage at -1.5 MPa varied from 62 to 91% at the suboptimal temperature (10C) but only from 88-98% at the optimal temperature (20C). Similarly, seed mortality following incubation for a week at -2MPa at 10C varied from 18 to 98%, whereas at 20C under these conditions, mortality was uniformly high (98-100%). In contrast, however, mycelial growth rate was uniformly low at -1.5 Mpa at the suboptimal temperature (23-38 mm diameter at 14 days) but varied more at optimal temperature (39-74 mm diameter at 14 days). The ability to kill seeds at 10C was weakly correlated with pathogen growth rate but not with conidial germination speed. The slowest-growing strain (23 mm at 14 days) caused by far the lowest seed mortality (18%) at 10C. These trials focused on dormant seed mortality at intermediate pathogen inoculum levels. The results suggest that selection for strains that perform better under suboptimal conditions could improve biocontrol efficacy of this organism, but much more work would be needed to determine the heritability and stability of these responses.

Objective Three – Host Specificity across a Suite of Invasive Annual Grasses

Our cross-inoculation trials with strains of *Pyrenophora semeniperda* from the seed banks of different annual grass weeds (Beckstead et al. 2016) yielded the following information. Host species differed in susceptibility to the pathogen as dormant seeds at relatively low inoculum loads, with seeds of red brome (*Bromus rubens*) the most resistant and those of rigput brome (*Bromus diandrus*) the least resistant. Seeds of cheatgrass (*Bromus tectorum*) and

medusahead (*Taeniatherum caput-medusae*) showed intermediate resistance, while those of Japanese brome (*Bromus arvensis*, formerly *Bromus japonicus*) yielded ambiguous results because the seeds of the particular lot used in the experiments were not fully dormant even when recently harvested.

Pathogen strains also varied in aggressiveness or virulence, but most of this variation was among strains within pathogen populations. There was no evidence for host specialization; pathogen strains did not cause higher levels of disease on their host of origin. Instead we found that the variability in aggressiveness exhibited by different strains was similar on different hosts, giving rise to positive correlations between aggressiveness on the host of origin and aggressiveness on the alternate host. These results are positive from the point of view of biocontrol because they indicate that there will be no advantage to trying to select host-specific strains for use as biocontrol agents against different annual grass weeds. Instead, a strain aggressive on one host is likely to also be aggressive on alternative hosts, simplifying the search for effective strains.

We also learned that a key result we obtained earlier for *B. tectorum* also applied to the other annual grasses in this study, namely that susceptibility to infection was similar for dormant and nondormant seeds, but tolerance to infection, as exhibited by the ability to escape through germination post- infection, was much higher in nondormant seeds.

We used two sets of neutral markers, single sequence repeats (SSRs) and the internal transcribed spacer sequence (ITS) of ribosomal DNA, to examine the population genetic structure of the pathogen on different hosts. We did detect some population differentiation, with the two marker systems yielding similar results. Pathogen populations from *B. tectorum* and the closely related species *B. rubens* and *B. diandrus* (all of the *Bromus* Section *Genea*) were also more closely related to each other than to pathogen populations from the more distantly related *B. arvensis* or *T. caput-medusae*. We determined that this differentiation was not an artifact of geographic origin within the introduced range. We concluded that this weak differentiation was probably due to founder effects. It is likely that seeds of these species arrived in the introduced range already contaminated with spores of their own pathogen populations. Eventually the lack of host specialization will likely break down this weak genetic differentiation, but only to the extent that different hosts are likely to co-occur in mixed populations.

Additional Key Finding – Capacity for Pathogen Sexual Reproduction

The discovery of major variation in mycelial growth rate and related traits associated with attack on host seeds in different dormancy states caused us to think about the idea of carrying out artificial breeding and selection for traits that would be more efficacious in strains of the fungus for biocontrol. This led us to spend considerable effort investigating the sexual life cycle of the organism in an effort to trigger sexual reproduction and collect recombinant genotypes for screening. The sexual state of the fungus is apparently rare in nature though it has been described, and it has only been reported a single time in culture. Most of the time this organism reproduces asexually, that is, clonally, producing progeny genetically identical to the parent and to each other. We had already determined using neutral markers that populations of the fungus were highly genetically diverse and exhibited low levels of linkage disequilibrium,

which argued that sexual recombination must occur regularly in nature even though it has rarely been observed.

As in other ascomycetes, the sexual system of this fungus involves two mating types represented in the genome by contrasting genetic forms or idiomorphs, which must both be present in order for sexual reproduction to occur. We began our studies by using our annotated genome of this organism to identify and sequence these two MAT idiomorphs (Henry 2015). We then genotyped our extensive strain collection to determine how these idiomorphs were distributed, both within strains and within and among populations. In general there are two types of mating systems in the ascomycetes, the heterothallic system, in which each strain contains a single MAT idiomorph, and the homothallic or self-fertile system, in which the two MAT idiomorphs occur in tandem on a single chromosome within the nucleus of a single strain.

We discovered that this fungus is heterothallic in the strict sense, in that the two MAT idiomorphs never occur in tandem on the same chromosome within the same nucleus. However, we did discover many strains that apparently contained both MAT idiomorphs, suggesting that these strains carried more than one nuclear type. This is not an uncommon condition in the ascomycetes—it comes about through hyphal fusion of vegetatively compatible strains. This compatibility has nothing to do with sexuality per se, but it results in strains that contain two nuclear types, and sometimes these two nuclear types can contain contrasting MAT idiomorphs, resulting in a condition referred to as pseudohomothallism. We independently demonstrated that strains with both MAT idiomorphs were also polymorphic for neutral markers, supporting the conclusion that they were heterokaryotic products of the hyphal fusion of genetically distinct but vegetatively compatible strains. Overall, we found that the two MAT idiomorphs were present in approximately equal proportions within most populations and also across all populations, which was further evidence that sexual reproduction was likely to occur regularly in nature.

Another emphasis of our work with the sexual system of the fungus was to explore whether the MAT idiomorphs were in fact functional to participate in sexual reproduction, that is, that they still contained unaltered the highly conserved portions most essential to the mating process. We discovered a high degree of polymorphism within the MAT loci themselves, but this polymorphism apparently did not disrupt the function of these genes in terms of any maladaptive modification of the genetic sequence (Henry 2015).

Once we had pairs of strains of known contrasting MAT genotype, we undertook the effort to persuade them to reproduce sexually by trying a very large number of permutations of cultural conditions that were thought to induce the sexual cycle in related fungi. Eventually we did obtain a few perithecia that apparently contained asci and ascospores, but we were unable to demonstrate genetically that these ascospores were actually recombination products of the parents. This was largely due to inexperience and poor handling of the material rather than any actual problem with the crosses themselves, but it did leave us without any recombinant strains to evaluate in terms of the traits of their parent strains. We also found a few perithecia on seeds collected from field seed banks, and these seemed to contain the same kind of spores that we had produced from our crosses in culture. However, these were not useful for breeding purposes, as their parents were unknown.

Our effort to breed this pathogen to produce more efficacious strains for biocontrol therefore failed in the short term, though we did learn a lot about the sexual cycle in this fungus that could still be useful for future research along these lines.

Additional Key Finding – Selection for Host General Resistance

We conducted field inoculation trials at the Whiterocks study site in Skull Valley, Utah, as well as at Haven Flats on the Hanford Reach National Monument over several years from 2008 through 2012, prior to the initiation of the set of studies described above for 2012-2015. We had originally proposed to carry out the studies under the current funding at these two sites as well as a third site in southern Idaho. However, on examining our results from Whiterocks, we realized that we had never been able to achieve the near-complete control at this site that we had achieved at several other sites. Rather than repeat these apparently futile efforts there, we decided to carry out a series of studies to test the hypothesis that selection for general resistance of dormant seeds to pathogen attack at low inoculum loads was leading to dominance of resistant cheatgrass genotypes in the Whiterocks carryover seed bank.

In the first study, we produced seeds of cheatgrass host lines characteristically found in specific habitats in the Intermountain range of cheatgrass (Merill et al 2012, Eldon 2013, Meyer et al. 2016) and inoculated them as dormant seeds with low levels of two strains from the Whiterocks pathogen population. We found that genotypes from salt desert and sagebrush steppe habitats had significantly higher resistance to pathogen attack under these conditions and consequently lower seed mortality than genotypes from warm desert and montane habitats (Meyer and Clement 2014). In general, host resistance was positively correlated with pathogen abundance across habitats and sites. The pathogen has been found at generally low abundance in cheatgrass seed banks in montane and warm desert habitats, and these were the habitats where the common cheatgrass lineages were least resistant. In contrast, cheatgrass lineages from cold desert and salt desert sites, where the pathogen is abundant, tended to have higher resistance and therefore lower mortality in the test.

These results supported the idea of selection for resistance in a general way, but there were many exceptions. For example, at Haven Flats, we achieved near-complete control using field inoculation, yet this site is not so different ecologically from the Whiterocks site, though it does have generally lower levels of the pathogen in the seed bank. In a more elaborate test for selection for general resistance at these two sites, we collected seeds from the current-year stand and from the carryover seed bank at each site, produced 100 plants from each of these four sample pools, and genotyped the plants using our newly developed SNP marker system. We then chose several sets of genetically uniform lineages that were over-represented either in the current year population or the population grown from the carryover seed bank at each site.

The hypothesis was that resistant lineages should be over-represented in the seed bank relative to the current year stand, because these seed bank seeds had survived pathogen attack in the soil, whereas the seeds collected from current year plants had never been exposed to the disease. Moreover, we predicted that this differential effect would be much stronger at Whiterocks than at Haven Flats for the following reason. Cheatgrass die-off results in failure of the current year stand, so that the only plants recruited locally in subsequent years must come from the carryover seed bank and therefore be survivors of *P. semeniperda* attack. Stand failure

is known to be a common occurrence at Whiterocks but has never been observed on a large scale at Haven Flats. We therefore predicted that selection for resistance to the pathogen should be much stronger at Whiterocks, and could be the reason for the poor results of our field inoculations there.

We tested our hypothesis by producing and inoculating dormant seeds of selected genetically uniform lineages over-represented in the current year stand and in the seed bank at each site and scoring subsequent seed mortality. At Haven Flats there was no evidence for selection for increased resistance, as lineages common in the current year stand and in the seed bank showed similar intermediate levels of resistance (25-30% mortality). But at Whiterocks, we saw dramatically higher resistance in lineages that were over-represented in the seed bank, indicating that they were indeed over-represented because they had been selected for resistance to the pathogen. Lineages from the carryover seed bank averaged less than 10% mortality, whereas those from the current year stand averaged 30-60% mortality.

These results indicate that one reason we have not been able to obtain complete mortality of the carryover seed bank at Whiterocks is that our field inoculum has not been applied at levels high enough to overcome this evolved resistance. We know that at high loads in the laboratory, seeds of all genotypes of the host can be killed. In order to achieve this desirable outcome in the field even in places with die-off histories and resulting high frequencies of resistant genotypes, we need to develop an inoculum delivery system that can deliver the high loads needed to kill even these resistant seeds.

IV. Management Implications

In the battle against annual grass weeds and their devastating effects on semiarid ecosystems in the Interior West, we know we need to use all weapons in the arsenal in an integrated management approach in the context of restoration seeding. While *P. semeniperda* does not promise to be the magic bullet that can solve this complex management problem, we still believe that it could be developed as a useful biocontrol tool for use in combination both with other biocontrols and with chemical or cultural control methods. We now have a very good understanding of the basic biology of this pathosystem and how it functions in a regulatory way in field seed banks. The work that needs to be done now is moving outside the realm of basic research and into the realm of technology development. In order to move forward it will be necessary to partner with industry to find better ways to produce and apply pathogen bulk inoculum for maximum effectiveness at minimum cost on a larger scale.

V. Relationship to Other Recent Findings and Ongoing Work on This Topic

Over the last twenty years we have worked extensively with several cheatgrass pathogens with the goal of finding some way to use these organisms for biocontrol. Most recently we have focused on the organisms responsible for cheatgrass stand failure or ‘die-off’ under natural conditions. Obviously an understanding of these organisms could lead to a breakthrough in biocontrol, as stand failure is essentially complete control of the current year stand, similar to what is achieved with pre-emergent herbicides. While many of the pathogens that strongly affect cheatgrass are not host-specific, we now know that we can successfully seed native grasses the year after a die-off and obtain superior stand establishment. This positive effect is due to the fact

that stand failure not only eliminates most competition from cheatgrass but also creates a fallow condition with surplus soil water and nitrogen that makes an ideal setting for a successful grass seeding. The pathogens that cause die-off are usually not very active the following year, for reasons we are still examining. The degree to which cheatgrass is controlled the year after a die-off also depends on the status of the carryover seed bank. At least a weak stand of cheatgrass usually establishes, but sometimes die-offs persist for multiple years. The key variable in whether the carryover seed bank is large enough to create serious competition post-die-off is the abundance of *P. semeniperda*. This brings us full circle on the need not only to understand and manipulate the die-off organisms themselves, but also to be cognizant of the role of *P. semeniperda* in die-off recovery rates and the length of the window of opportunity for restoration seeding.

VI. Future Work Needed

In order to effectively use *P. semeniperda* as a biocontrol for eliminating the carryover seed bank of weedy annual grasses, we still need to develop a bulk inoculum technology that results in a lightweight product of consistently high potency, so that we can realistically apply inoculum loads that are sufficient to eliminate viable annual grass seeds under a wider range of environmental conditions. We plan to continue to pursue this possibility, in conjunction with current research on other fungal pathogens and rhizobacteria that can kill actively germinating annual grass seeds and prevent stand establishment or severely suppress their growth as an alternative to the use of imazapic and other chemical herbicides. We also plan to pursue the idea of breeding more efficacious strains of the fungus and also of using strain mixtures to increase overall biocontrol success.

VII. Deliverables Crosswalk Table

Deliverable	Description	Delivery Date
Technical meetings and tour presentations	1 webinar for Great Basin Science Delivery series	3/2012
National/international meetings/presentations	14	See list.
Regional presentations	2 invited	11/2014; 2/2016
Master's theses	4 master's theses	2013 (2), 2014 (1), 2015 (1)
Peer-reviewed publications	7 published peer reviewed manuscripts based on previous round of JFSP funding (published post-final-report); 9 published peer reviewed manuscripts based on current round of JFSP funding.	2011-present
Final report	Summarized findings in a final report	3/30/2016

Please see the list of publications and presentations (Section VIII below) for details.

VIII. Publications and Presentations

Peer-reviewed Publications from JFSP Project Number 2007-1-3-10 (2007-2010) (published after submission of final report):

- Baughman OW, and Meyer SE. 2013. Is *Pyrenophora semeniperda* the cause of downy brome (*Bromus tectorum*) die-offs? *Invasive Plant Science and Management* 6:105–111.
- Beckstead J, Street LE, Meyer SE, and Allen PS. 2011. Fire effects on the cheatgrass seed pathogen *Pyrenophora semeniperda*. *Rangeland Ecology and Management* 64:148-167.
- Beckstead J, Miller LE, and Connolly BM. 2012. Direct and indirect effects of plant litter on a seed pathogen interaction in *Bromus tectorum* seed banks. *Seed Science Research* 22:135-144.
- Beckstead J, Meyer SE, Reinhart KO, Bergen KM, Dooley SR, and Boekweg H. 2014. Factors affecting host range in a generalist seed pathogen of semi-arid shrublands. *Plant Ecology* 215:427-440.
- Boose D, Harrison S, Clement S, Meyer SE. 2011. Population genetic structure of the seed pathogen *Pyrenophora semeniperda* on *Bromus tectorum* in western North America. *Mycologia* 103:85-93.
- Merrill KR, Meyer SE, Coleman CE. 2012. Population genetic analysis of *Bromus tectorum* (Poaceae) indicates recent range expansion may be facilitated by specialist genotypes. *American Journal of Botany* 99:529-537.
- Meyer SE, Merrill KT, Beckstead J, and Allen PS. 2014. Indirect effects of a seed bank pathogen on the interactions between *Bromus tectorum* and two native perennial grasses. *Oecologia* 174:1401-1413.

Peer-reviewed Publications from JFSP Project Number 2011S-2-6 (2011-2015)

- Barth C, Meyer SE, Allen PS, Beckstead J. 2015. Hydrothermal time models for conidial germination and mycelial growth of the seed pathogen *Pyrenophora semeniperda*. *Fungal Biology* 119:720-730.
- Beckstead J, Meyer SE, Ishizuka TS, McEvoy KM, and Coleman CE. 2016. Lack of host specialization on winter annual grasses in the fungal seed bank pathogen *Pyrenophora semeniperda*. *PLoS ONE* 11(3): e0151058. doi:10.1371/journal.pone.0151058
- Finch, H, Allen PS, and Meyer SE. 2013. Environmental factors influencing *Pyrenophora semeniperda*-caused seed mortality in *Bromus tectorum*. *Seed Science Research* 23:57-66.
- Masi M, Evidente A, Meyer S, Nicholson J, and Muñoz A. 2014a. Effect of strain and cultural conditions on the production of cytochalasin B by the potential mycoherbicide *Pyrenophora semeniperda* (Pleosporaceae, Pleosporales). *Biocontrol Science and Technology* 24:53-64
- Masi M, Meyer SE, Clement S, Andolfi A, Cimmino A, and Evidente A. 2014b. Spirostaphylotrichin W, a spirocyclic β -lactam isolated from liquid culture of *Pyrenophora semeniperda*, a potential mycoherbicide for cheatgrass (*Bromus tectorum*) biocontrol. *Tetrahedron* 70:1497-1501.
- Masi M, Meyer SE, Cimmino A, Andolfi A, and Evidente A. 2014c. Pyrenophoric acid, a new phytotoxic sesquiterpene produced by *Pyrenophora semeniperda*, a potential mycoherbicide for *Bromus* biocontrol. *Journal of Natural Products* 77:925-930.

- Masi M, Meyer S, Cimmino A, Clement S, Black B, Evidente A. 2014d. Pyrenophoric acids B and C, two new phytotoxic sesquiterpenoids produced by *Pyrenophora semeniperda*. *Journal of Agricultural and Food Chemistry* 62:10304-10311.
- Meyer SE, Masi M, Clement S, Davis T, Beckstead J. 2015. Mycelial growth rate and toxin production in the seed pathogen *Pyrenophora semeniperda*: Resource trade-offs and temporally varying selection. *Plant Pathology* 64:1450-1460.
- Meyer SE, Leger EA, Eldon DR, Coleman CE. 2016. Strong genetic differentiation in the invasive annual grass *Bromus tectorum* across the Mojave-Great Basin ecological transition zone. *Biological Invasions*. [DOI 10.1007/s10530-016-1105-6](https://doi.org/10.1007/s10530-016-1105-6)

Masters Theses from JFSP Project Number 2011S-2-6 (2011-2015):

- Finch, Heather. 2013. The *Bromus tectorum* – *Pyrenophora semeniperda* pathosystem. 2013. Master's of Science Thesis, Brigham Young University, Provo Utah.
- Hawkins, Katie Karen. 2014. Secondary dormancy and summer conditions influence outcomes in the *Pyrenophora semeniperda* - *Bromus tectorum* pathosystem. Master's of Science Thesis, Brigham Young University, Provo Utah.
- Eldon, Desiree Rochelle. 2013. Population genetic structure of *Bromus tectorum* in the American Desert Southwest. Master's of Science Thesis, Brigham Young University, Provo Utah.
- Henry, Julie Leanna. 2015. Mating type locus characterization and variation in *Pyrenophora semeniperda*. Master's of Science Thesis, Brigham Young University, Provo Utah.

Presentations (National and International Meetings):

- Beckstead J, KT Merrill, SE Meyer, PS Allen. 2011. Cheatgrass effects on native grass seed and seedling fate: competition, facilitation, and indirect effects of a shared seed pathogen. COS-43-4 Ecological Society of America 97th Annual Meeting, August 7-12, 2011, Austin TX.
- Finch H, SE Meyer, PS Allen. 2011. How the seed bank pathogen *Pyrenophora semeniperda* kills nondormant cheatgrass seeds. COS-11-5. Ecological Society of America 97th Annual Meeting, August 7-12, 2011, Austin TX.
- Meyer SE, KR Merrill, SJ Novak, EA Leger, and CE Coleman 2011. Population genetic structure of *Bromus tectorum* in western North America; Implications for the invasion of novel habitats. COS-31-8. Ecological Society of America 97th Annual Meeting, August 7-12, 2011, Austin TX.
- Meyer SE, J Beckstead, and PS Allen. 2011. Development of a fungal seed bank pathogen for cheatgrass biocontrol on Intermountain rangelands. Society for Range Management 64th Annual Meeting, February 6-10, 2011. Billings, Montana.
- Beckstead J, SE Meyer, and PS Allen. 2012. A naturally-occurring seed pathogen eliminates the cheatgrass carryover seed bank in the field. Society for Range Management 65th Annual Meeting, January 29-February 3, 2012, Spokane WA.

Meyer SE. 2012. Are cheatgrass die-offs in the Great Basin an opportunity for long-term control? Society for Range Management 65th Annual Meeting, January 29-February 3, 2012, Spokane WA.

Allen PS, SE Meyer, and J Beckstead. 2013. A predictive model for soil seed bank outcomes in the *Pyrenophora semeniperda*-*Bromus tectorum* pathosystem. First International Conference on Wild Plant Pathosystems, July 2-5, 2013, Olomouc, Czech Republic.

Finch H, PS Allen, and SE Meyer. Exposure to low water potentials and seed dormancy favor the fungus in the *Pyrenophora semeniperda*-*Bromus tectorum* pathosystem. First International Conference on Wild Plant Pathosystems, July 2-5, 2013, Olomouc, Czech Republic.

Hawkins K, PS Allen, and SE Meyer. Secondary dormancy of seeds in relation to the *Bromus tectorum*-*Pyrenophora semeniperda* pathosystem. First International Conference on Wild Plant Pathosystems, July 2-5, 2013, Olomouc, Czech Republic.

Beckstead J, T. Ishizuka, K. McEvoy, and SE Meyer. 2014. Pathogen-caused seed mortality among a suite of annual weeds influenced by seed dormancy and inoculum load but not host specificity. Presentation 143. Botanical Society of America Botany 2014 Meeting, July 26-30, 2014, Boise ID.

Franke J, SE Meyer, and B Geary. 2014. Bleache blonde syndrome: a new disease of *Bromus tectorum* implicated in cheatgrass die-offs. Presentation 446. Botanical Society of America Botany 2014 Meeting, July 26-30, 2014, Boise ID.

Grome K, J Beckstead, K Van Volkom, and A Shuster. 2014. Residual effects of imazipic on the invasive cheatgrass and native species in a restoration setting. Presentation 278. Botanical Society of America Botany 2014 Meeting, July 26-30, 2014, Boise ID.

Ledell B, J Beckstead, and K Van Volkom. 2014. Restoration opportunity: Understanding the effects of naturally occurring pathogens on native and nonnative grasses under water stress conditions. Presentation 279. Botanical Society of America Botany 2014 Meeting, July 26-30, 2014, Boise ID.

Masi M, SE Meyer, S Clement, A Cimmino, A Andolfi, and A Evidente. 2014. Phytotoxin production by the pathogen *Pyrenophora semeniperda* and its possible role in pathogenesis on *Bromus tectorum* seeds. Presentation 168. Botanical Society of America Botany 2014 Meeting, July 26-30, 2014, Boise ID.

Meyer SE, and S Clement. General resistance in the *Bromus tectorum*-*Pyrenophora semeniperda* pathosystem. Presentation 143. Botanical Society of America Botany 2014 Meeting, July 26-30, 2014, Boise ID.

Presentations (Regional and Technical Meetings):

Boyte, S, and SE Meyer. 2012. Cheatgrass die-off in the Great Basin: Quantifying spatial extent and potential causal mechanisms. Webinar, Great Basin Fire Science Exchange, Thursday, March 15, 2012.

Meyer SE. 2014. Cheatgrass die-off as a restoration opportunity. 2014. Invited presentation. The Next Steppe Conference, November 5-7, 2014, Boise ID.

Meyer SE, J Beckstead, J Franke-Pearce. 2016. Fungal pathogens on *Bromus tectorum*: Ecology and management. Invited presentation. Sagebrush Ecosystem Conservation: All Hands, All Lands. February 23-26, 2016, Salt Lake City UT.