Project Title: Annual Brome Biocontrol after Wildfire Using a Native Fungal Seed Pathogen

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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. These seeds can provide significant competition for seeded species in the years following treatment. We investigated the feasibility of using a naturally occurring seed pathogen, the ascomycete *Pyrenophora semeniperda*, as a biocontrol organism for eliminating this carryover seed bank. We carried out the necessary technology development to create and apply field inoculum to cheatgrass- or red brome-infested areas (both burned and unburned) at six sites located in three states across two years of field trials. We found that inoculum application significantly increased the proportion of pathogen-killed *Bromus* seeds in the seed bank, reduced the density of viable carryover *Bromus* seeds, and in many cases increased the density of pathogen-killed seeds relative to levels in uninoculated controls. In some treatments, the proportion of field-killed seeds reached 100%, validating the promise of this approach. Even though this pathogen is physiologically capable of infecting the seeds of many grasses and some dicots, we determined that risks to nontarget host seeds can be mitigated. The inoculum usually has a relatively short persistence time in the absence of new host seeds, and the pathogen is readily controlled by fungicides that could potentially be used as seed treatments for desired restoration species. The potential for selection of more virulent *P. semeniperda* strains for increased biocontrol effectiveness is considerable. In addition, because more virulent strains grow more slowly, they are less likely to persist post-control in competition with faster-growing wild strains. In summary, our study provides proof of concept for use of this pathogen for biocontrol of cheatgrass and red brome, and opens the way for further studies on formulation and delivery technology to bring this promising biocontrol agent closer to market.

II. Background and Purpose

A major obstacle to seeding success with native species as part of post-burn rehabilitation in arid shrubland ecosystems is competition from exotic annual brome grasses such as cheatgrass (*Bromus tectorum*) and red brome (*Bromus rubens*). In many cases, these are the same grass species that fueled the shrub-destroying fire. Seed banks of annual bromes are depleted but usually not completely destroyed by burning (Beckstead *et al.* in press). The common wisdom is to seed as quickly as possible after the fire that destroys the shrub overstory, in order to give the seeding a chance to establish before annual brome competition builds back up. In arid ecosystems, seedings often fail because of inadequate precipitation, and this window of opportunity closes quickly. Once annual bromes reestablish dominance after the shrub-destroying fire, it becomes very difficult to seed successfully on these sites, even if they reburn. This is because more brome seeds survive fire when hot-burning woody fuels are no longer present. If we could find a way to destroy the residual annual brome seed bank after fire, the probability of successful rehabilitation would

be greatly increased, and even sites that have burned many times and are in persistent annual brome monocultures could perhaps be seeded successfully.

The options available for control of annual brome grasses in arid wildland ecosystems are limited, and each has disadvantages. Early season burning, before seed dispersal, can eliminate most seeds, but there may still be carryover seeds in the seed bank. Prescribed burning in these ecosystems is risky and raises other issues, such as air quality. Tillage after annual brome emergence is too expensive to undertake on large acreages, damages remnant perennials, and causes soil disturbance. Herbicides clearly have a place in the arsenal against annual grass weeds, but tend to be expensive. The detailed, habitat-specific research needed to understand herbicide impact on non-target species is often not in place to guide management. There are many policy issues surrounding the use of herbicides as well.

One problem common to all these control methods is that they do little or nothing to eliminate ungerminated seeds. Annual grass weeds respond dramatically to increases in available resources. This means that even a few ungerminated carryover seeds per unit area have the potential to quickly reestablish a population. Effective control for these grass weeds must include elimination of this bank of ungerminated seeds. A biocontrol option that targets the ungerminated seeds of annual grass weeds specifically in the context of post-fire rehabilitation would therefore be a very useful tool in the restoration toolkit. In this study, we investigated the potential for the naturally occurring seed pathogen *Pyrenophora semeniperda* (Ascomycota; imperfect state *Drechslera campanulata*) to become this tool. This pathogen is already abundant in many annual brome seed banks and can cause high mortality of carryover seeds even at inoculum levels commonly observed in the field.

In conducting this research, we focused on three study objectives:

1) Experimentally determine the effectiveness of the pathogen as a biocontrol organism, alone and in combination with other control measures.
2) Evaluate risks to non-target organisms, including seeded species.
3) Develop strategies for minimizing identified risks.

III. Study Descriptions and Locations

1) Pathogen Host Range Experiments

Seeds for a wide range of species (>50) that currently occupy semiarid ecosystems of western North America were subjected to *P. semeniperda* inoculation under laboratory conditions. Seeds were challenged with inoculum from two pathogen strains in a series of laboratory experiments, either as conidia (Petri dish experiments) or in ring samples of field seed banks (see Beckstead *et al.* 2010 for methods) that contained natural levels of the fungus. These species included annual bromes and other common weeds, as well as species used by land management agencies for post-fire rehabilitation in annual brome-infested wildlands. In addition, disease levels in *in*

situ seedbanks of *Bromus tectorum* and native grasses were quantified in both Utah and Washington.

2) Laboratory Virulence Screening

The objective of virulence screening was to examine variation in pathogen virulence as a prelude to possible selection for higher virulence in strains to use for biocontrol. The first step was to obtain viable conidial inoculum from multiple pathogen strains. This involved isolating strains from infected cheatgrass or red brome seeds obtained from the field as part of seed bank evaluation at 24 sites and placing these isolates under conditions conducive to conidial production. We spent the first 18 months of our study developing the techniques necessary to achieve this goal (Stewart 2009; Stewart *et al.* 2009). In spring 2009 we performed the first large scale virulence screening test, with 90 isolates, 40 of which had conidia of sufficiently high viability to be included in the final analysis (Stewart *et al.* 2009). In fall 2009 we performed virulence screening on an additional 38 strains with high conidial viability.

We also measured mycelial growth rate for a subset of 20 isolates from each virulence screening test, for a total of 40 isolates. The purpose of the growth rate studies was to test the hypothesis that higher virulence would be associated with faster growth rate. We used analysis of covariance to relate virulence (% host seed mortality) to growth rate (14-day colony diameter).

3) Inoculum Production Technology Development

Bulk inoculum for field inoculation trials was produced by seeding conidia of selected pathogen strains into potato dextrose broth (PDB) in autoclaved large-batch (10 liter) glass fermenters and growing mycelium for 2-3 days at room temperature in aerated submerged culture. The resulting mycelial mass was concentrated by centrifuging and mixed with fresh PDB prior to mixing with a sterilized inert carrier (Agsorb calcined montmorillonite clay). The resulting material was dried slowly for 24-48 hours under cool white and ultraviolet lights to encourage conidial production on the carrier. Following sporulation, the material was further dried in covered containers in a warm greenhouse. It was then pressed through a sieve to break the material into granular form. The bulk inoculum was then weighed into vials for hand field application. This procedure was derived essentially through trial and error and not through any systematic variation in conditions of formulation; a more systematic approach to optimizing the formulation process is currently in progress.

4) Field *In Situ* Seed Bank Studies

In order to characterize annual brome seed bank dynamics and the role of the seed bank pathogen *Pyrenophora semeniperda* in those dynamics, we selected five study sites and took seed bank samples four times a year for two years (August 2007 – May 2008) at each site. Two of the sites (Pakoon AZ and Mormon Mountain NV) were red brome sites, while the other three (Whiterocks UT, Cinder Cone Butte ID, and Saddle

Mountain WA) were cheatgrass sites. At the Pakoon red brome site, we sampled from both unburned and burned areas, while at Mormon Mountain only a burned area was available. The three cheatgrass sites had not burned for at least 5 years. Seed bank sampling, processing, and analysis followed the protocols in Meyer *et al.* (2007). For each site, 20 seed bank samples were obtained on each date using a repeated measures design. These were hand-processed to quantify germinated seeds and seeds killed in the field by the pathogen, and apparently healthy seeds were then incubated for 4 wks at 20C and scored as germinable, dormant, killed by the pathogen in incubation, or nonviable for unknown reasons. We could then calculate the proportion of the total seed bank present in August that germinated, carried over as viable seeds, or was killed by the pathogen. We then compared these proportions among species, sites and years.

5) Field Inoculum Persistence Study

To address the question of how long pathogen inoculum can persist in the absence of host seeds, we placed inoculated cheatgrass seeds in six block replications of 20-seed packets in the field at three sites: mesic (Spokane WA), semiarid (Whiterocks UT) and arid (Pakoon AZ). We placed the inoculated seeds in nylon packets on the surface of seed-free and disease-free soil, in pots flush with ground level, then covered the packets with seed-free and disease-free litter and window screening. Over the course of three growing seasons, we periodically retrieved packets of seeds, removed pathogen stromata, and incubated them in the presence of dormant cheatgrass seeds to determine if these stromata were still capable of infecting and killing seeds. After the second growing season, we also cultured individual stromata from the original seeds to directly determine if they were capable of sporulation or growth.

6) Field Herbicide/Fungicide Trials

We conducted an experiment to evaluate the ability of three different fungicides (Headline, Indar and Rally) to reduce field inoculum loads of *P. semeniperda*. Following fungicide application in the field, we collected ring seed bank samples from treated and untreated areas and determined mortality of cheatgrass seeds planted into the rings (see Beckstead *et al*. 2010 for methods). We also completed experiments to determine whether glyphosate herbicide would have any effect on the ability of the fungus to kill cheatgrass seeds, by planting into ring samples from glyphosate-treated and control areas as described for the fungicide experiment above. In addition, we combined inoculation treatments with herbicide treatments in factorial combination in the first year of field inoculation trials at three sites (see below).

7) Field Inoculation Experiments

We tested the efficacy of laboratory-produced *P. semeniperda* bulk inoculum for reducing the density of the annual brome carryover seed bank in small plot field trials at five sites (Whiterocks UT, Haven Flats WA, Pakoon AZ, Davis Mountain UT, Santaquin Canyon UT) in 2008/2009, and three sites (Whiterocks UT, Haven Flats WA, and Lytle Ranch UT) in 2009/2010. Two of the sites (Pakoon and Lytle Ranch)

were red brome sites, and five of the trials (at Whiterocks, Haven Flats, and Pakoon) included burned and unburned treatments. The 2008/2009 trials at Whiterocks, Haven Flats, and Pakoon also incorporated a set of herbicide treatments, including the post-emergent herbicide RoundUp (glyphosate) and the pre-emergent herbicide Plateau (imazapic), to examine whether control of the carryover seed bank using the pathogen could be combined with the use of herbicides for control of establishing/ established plants. We applied bulk inoculum derived from different pathogen strains and at different application rates in August/September (after dispersal but before germination) and measured the densities of viable and killed seeds the following spring (after completion of germination but before dispersal) by taking seed bank samples from each plot and processing following protocols described in Meyer *et al.* (2007). We also measured biomass production on the plots by clipping aboveground biomass late during seed maturation, at the same time seed bank samples were taken. At the end of the summer following seed bank sampling and biomass harvesting, we obtained ring bioassay samples from representative treatments (see Beckstead *et al.* 2010 for methods) and planted native grass seeds into these to determine the effect of residual inoculum on native grass (bluebunch wheatgrass and squirreltail) emergence. Residual inoculum studies were carried out at Whiterocks (2 years), Haven Flats (2 years), and Lytle Ranch (1 year).

IV. Key Findings

1) Pathogen Host Range Experiments

Laboratory inoculation trials demonstrated that of the 50+ species tested to determine susceptibility to *Pyrenophora semeniperda*, most were at least somewhat susceptible to infection when challenged with high loads of inoculum. However, species varied greatly in both susceptibility to infection and mortality following infection. Seed mortality was primarily a function of germination rate (Beckstead *et al.* 2008, 2009, in preparation). Fast-germinating seeds tended to escape mortality even when successfully infected, while slower-germinating seeds were either killed following infection or possessed mechanisms to resist infection. At decreasing inoculum loads, mortality of native grasses dropped more quickly than for cheatgrass (*Bromus tectorum*), which is a positive finding from the standpoint of further development of this fungus as a mycoherbicide. Sampling of soil seed banks associated with native perennial grass communities revealed the striking near-complete absence of both native seeds and *P. semeniperda* in seed banks in these communities. Inoculation treatments that successfully reduced the carryover seed banks of cheatgrass and red brome produced minimal mortality to seeds of native species (bluebunch wheatgrass and squirreltail) that were seeded one year following inoculum application. These results suggest that application of the fungus may pose little threat to native plant species in either intact communities or under conditions where restoration seeding would be timed to occur the year following biocontrol of annual bromes.

2) Laboratory Virulence Screening

While almost all pathogen strains were capable of killing dormant cheatgrass seeds in laboratory trials, pathogen virulence, as measured by the ability to kill nondormant cheatgrass seeds, varied over a wide range, from 0 to 44% (Meyer *et al.* 2010). This variation indicates that it might be possible to select for more virulent strains that would be more efficacious in biocontrol. The strains included in two independent virulence screening tests showed similar near-normal virulence frequency distributions, with most isolates falling in the intermediate categories (11-25% host seed mortality). Only a few isolates caused host seed mortality higher than 30%, indicating that high virulence might be associated with potentially maladaptive traits.

We had hypothesized that virulence and growth rate should be positively correlated, based on the idea that, if faster-germinating seeds are more likely to escape (Beckstead *et al.* 2007), then faster-growing isolates should be more likely to stop their germination and kill them. We actually obtained the opposite result—more virulent strains grew significantly more slowly than less virulent strains (Meyer *et al.* 2010). This pathogen is a necrotroph, which means that it causes pathogenesis through the production of toxins. Our current hypothesis is that the production of these toxins is metabolically expensive, so that highly virulent strains have fewer resources left for growth and therefore grow more slowly. This creates the interesting possibility that, if we can select or possibly breed a highly virulent strain, it might grow so slowly that, once it does its job of eliminating the cheatgrass seed bank, it could fail to persist in competition with less virulent but faster growing wild pathogen strains. This could be a major advantage in developing a strain for biocontrol, as the potential risk to nontarget host seeds could be significantly reduced if a highly virulent strain is less likely to be persistent.

3) Inoculum Production Technology Development

As discussed above under study descriptions, we have approached the problem of production of pathogen bulk inoculum largely through trial and error, yet the bulk inoculum we produced was able to kill large numbers of seeds in field inoculation trials (see below). This indicates that with further, more systematic optimization of formulation and carrier delivery technology, this biocontrol agent could be extremely effective against the carryover seed banks of annual bromes.

4) Field *In Situ* Seed Bank Studies

Densities of both viable and killed seeds in annual brome seed banks changed predictably during the year, but absolute values varied widely as a function of brome species, site and year (Meyer et al. 2009, in preparation). For cheatgrass, seed densities in August (after dispersal but before any germination) varied from about 6,000 to 25,000 seeds-m^{-2}, while for red brome, these values were lower (3,000-16,000 seeds-m^{-2}), probably due a slow buildup after severe drought in 2006 and 2007, when few or no seeds were produced. From 40-70% of the cheatgrass seeds

present in August germinated during the following autumn/spring, while 3-35% successfully carried over and 10-53% were killed by the pathogen. Red brome seed banks followed a somewhat similar pattern, except germination percentage was generally higher (36-94%) and successful carryover percentage much lower (3-9%) than for cheatgrass. Pathogen-caused mortality on red brome seeds varied widely, from a low of 3% at Mormon Mountain the first year to a high of 56% in the unburned area at Pakoon the second year. In general, red brome seeds successfully carry over only in dry years, while cheatgrass has physiologically induced secondary dormancy to facilitate substantial seed bank carryover each year (Allen and Meyer 2010). In summary, the pathogen *P. semeniperda* is an important player at naturally occurring inoculum levels in the seed banks of both cheatgrass and red brome, but at least some successful seed bank carryover usually occurs at these inoculum levels.

5) Pathogen Inoculum Persistence Study

Because we placed infected seeds in the field in autumn, we expected the pathogen in these seeds to sporulate and produce conidia the following spring, and these that conidia would germinate at the first rainfall opportunity. Our question was: what happens to pathogen stromata after they have produced conidia—how long do they live, and are they still competent to infect seeds? Using the ability to infect adjacent dormant seeds as a test, we detected a tendency for these stromata to lose viability during the two years following conidial production more quickly at the most mesic site (Spokane WA) than at the most xeric site (Pakoon AZ). However, contamination by *in situ*-produced conidia prevented us from drawing clear conclusions using this method. By directly measuring the ability of stromata from the retrieval bags to grow in culture and/or produce conidia, we found that the mesic and semiarid sites had <10% viable stromata after one year post-conidial production in the field, while the xeric site had >95% viable stromata after one year and >75% after two years. These results suggest that stromata deteriorate rapidly under moist conditions once they have exhausted seed resources and produced conidia, but that they may persist as resting structures for at least two subsequent years under dry conditions. Whether and how these resting structures infect new seeds is not known.

6) Field Herbicide/Fungicide Trials

All three fungicides we tested dramatically reduced levels *P. semeniperda* inoculum in the field, as measured by ring bioassay experiments with dormant cheatgrass seeds. These results were expected, and suggest that fungicides can be used if necessary in order to facilitate successful seeding of desirable species. Control increased linearly with application rate for all three fungicides, indicating that high application rates would be necessary for complete control. This is probably because these fungicides were developed primarily for use against foliar pathogens (including related *Pyrenophora* spp.) and may be quickly immobilized in soil. Application directly to the seeds that require protection rather than as a drench to soil would probably greatly reduce the required application rates.

Glyphosate herbicide effectively controlled emerged cheatgrass seedlings, but had no effect on the pathogen. In further field trials (see next section), there were no significant interactions between pre-and post-emergent herbicides and fungal inoculum treatments. Lack of an herbicide effect on the fungus suggests that the two treatments could be used in combination.

7) Field Inoculation Trials

Application of laboratory-produced bulk inoculum of *P. semeniperda* in field inoculation trials consistently reduced the proportion of viable seeds and increased the proportion of killed seeds in the potential carryover seed bank (Allen *et al.* in preparation). Killed seed proportion generally increased with increasing inoculum load. At the highest loads, kill proportion averaged 89%, which represented a mean increase of 35% over background disease levels, which averaged 54%. The kill proportion reached 100% in some treatment combinations, suggesting that further optimization of the technology for formulation and delivery of this biocontrol agent could result in consistent near-complete removal of the carryover annual brome seed bank. Burning before treatment generally had little effect on the efficacy of the inoculation treatment, though it did decrease the total size of the potential carryover seed bank, making the goal of complete control more achievable.

Herbicide treatments in trials with Roundup (glyphosate) or Plateau (imazapic) gave essentially complete control of the emerging/emerged annual brome stand, but had no measurable effect on the efficacy of pathogen inoculum, indicating that these types of treatments could be successfully combined for annual brome control. Ring bioassays to evaluate the impact of residual inoculum a year after application showed a measurable but very small negative effect on emergence of two perennial grass species (reductions <15%).

8) Additional Key Findings

We initiated a series of studies to learn how both cheatgrass seeds in the seed bank and the fungal pathogen *P. semeniperda* respond to fire. Results from a thermal death point experiment showed that pathogen propagules were killed at a higher temperature than cheatgrass seeds, indicating that it would be better able to survive fire. Pyrometer measurements from controlled field burns indicated that temperatures high enough to kill seeds or pathogen propagules via radiant heat were rarely reached, especially if seeds or propagules were at the base of the litter or below the soil surface. Ring bioassays and seed bank sampling before and after fire supported these conclusions (Beckstead *et al.* in press).

The simultaneous use of multiple organisms for biocontrol may have unpredictable consequences. In order to characterize the interaction between *P. semeniperda* and a deleterious rhizobacterium (*P. fluorescens* D7) selected for virulence to *Bromus tectorum*, we designed a series of experiments to examine the possible interactions of these two organisms. We found a slight increase in fungal infection with dual inoculations but a decrease in fungal-caused seed mortality. We observed no increase

in the inhibitory effect of *P. fluorescens* with dual inoculations and in some cases saw less growth inhibition in the presence of both microorganisms. Overall, our findings suggest that there is no beneficial interaction between *P. semeniperda* and *P. fluorescens* D7 that would provide improved cheatgrass control (Dooley and Beckstead 2010).

We investigated the direct and indirect effects of litter on the interaction between the pathogen and cheatgrass seeds. We found that seed bank samples from high-litter patches contained higher field-killed seed densities compared with low-litter patches, although the magnitude of disease varied among sites and years. We also found that litter can act as a direct inoculum source for the pathogen in the early summer soon after production but that this effect decreases by the following spring, when the litter naturally is in contact with seeds. Investigating indirect effects, we found pathogen-killed seeds to be four times higher in high-litter treatments compared with low-litter treatments when inoculum loads and seed densities were held constant. In addition, we found that litter influenced the seed-pathogen interaction through density-dependent disease transmission. Our findings demonstrate the ecological importance of litter in semiarid environments in mediating disease levels of this seed pathogen by both direct and indirect means (Miller *et al.* 2009, Beckstead *et al.* in preparation).

A commonly observed phenomenon in the Great Basin where cheatgrass occurs as monocultures over large areas is 'die-off', or complete stand establishment failure. We examined whether *P. semeniperda* might play a role in this phenomenon by sampling seed banks in die-off areas and in adjacent control areas where a current-year stand was present at a series of ten die-off sites in western Utah and central Nevada. We found that the density of killed seeds was either the same in die-off areas and control areas, or lower in die-off areas than control areas. This was negative evidence on the causal role of this organism in the die-off phenomenon, but we found we could use the comparative seed bank data to estimate the age since die-off, as these areas do not produce new seeds. For recent die-offs (within one year), carryover seed banks in control and die-off areas were the same, whereas in older die-offs, the density of both viable and killed seeds was much reduced in the die-off areas relative to the control areas (Baughman and Meyer 2009, in preparation). We also isolated pathogen strains from the die-off areas and determined that they were no more virulent on average than strains from areas with extant cheatgrass stands.

IV. Management Implications

This study proved that biological control using the naturally occurring seed pathogen *P. semeniperda* holds considerable promise in the arsenal of tools that land managers need in order to combat annual bromes. While we do not yet have a product formulation for the fungus that can be made available commercially, the development of such a product remains a strong possibility with further research. Even though we achieved virtually complete control of *Bromus* carryover seeds in some trials, it is likely that a much more effective strain of the fungus may yet be discovered or created.

Based on our results so far, we believe that the pathogen will prove to be most useful as a mycoherbicide in locations where the carryover seed bank is most problematic (i.e., xeric sites). Where desired, this fungus can be used in conjunction with herbicides and its post-control effects can be mitigated by treatment with appropriate fungicides. It should also be possible to develop fungicidal seed treatments for susceptible restoration species, similar to treatments already in place for seeds of many crop plants. Alternatively, the risks to non-target species may be mitigated by seeding the year following biocontrol treatments.

This study also demonstrates that *P. semeniperda* occurs naturally at relatively high levels and already kills many seeds of cheatgrass and red brome in the field. Managers should be aware that inoculum loads could potentially be high enough at some sites and in some years to contribute to decreased success of restoration seedings, even without the addition of inoculum (Beckstead et al. 2010, Merrill et al. in preparation). However, once the carryover seed band is eliminated, persistence of viable pathogen propagules under field conditions is likely to be of short duration (less than one year) in all but the most xeric sites.

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

Our research group has also recently conducted extensive work on more basic aspects of the biology of *Pyrenophora semeniperda* and its cheatgrass host. We have used 454 pyrosequencing technology to sequence and assemble the genome of this pathogen, and have also used the sequence data we obtained to develop a series of molecular markers for population genetics work. These include ITS (internal transcribed spacer sequence from ribosomal DNA) markers, SSR (single sequence repeat or microsatellite) markers, and SNP (single nucleotide repeat) markers. These markers have been used to characterize our collection of over 700 isolates (Boose et al. in press, Boose et al. in preparation, Meyer et al. 2010). The patterns of population differentiation we have observed indicate that strains of the pathogen on cheatgrass and red brome may have arrived from Eurasia on the seeds of their annual brome hosts. This conclusion is supported by the fact that we were able to collect and identify the pathogen from cheatgrass seeds in Greece and Turkey, in spite of the fact that this pathogen was not previously known to occur in Eurasia (Stewart *et al.* 2009).

The population genetic data also strongly suggest that this organism undergoes regular sexual reproduction, with the accompanying recombination of neutral marker loci as well as the loci that control virulence (Meyer *et al.* 2010). We are exploring the genetics of virulence through annotation of an EST library developed from messenger RNA using 454 pyrosequencing, combined with biochemical characterization and quantification of the toxins found in strains that vary in virulence. This work may help us to select or breed for hypervirulent strains that will be more effective for biocontrol.

The discovery that growth rate and virulence are negatively correlated in this organism led us to ask what would happen if fast-growing and virulent strains were inoculated onto the same seeds. We used our SSR markers to fingerprint the stromata of co-infected

seeds, and determined that the virulent strain was more likely to successfully sporulate on rapidly germinating nondormant host seeds, while the fast-growing strain was more likely to successfully sporulate on dormant seeds (Davis *et al*. in preparation). These results support the idea that a hypervirulent strain developed for biocontrol would be outcompeted by less virulent but faster-growing strains on the dormant seeds that are the primary target of the pathogen.

One surprising result of our first round of field inoculations was a sometimes significant reduction in standing biomass at high inoculum loads, which suggested that the pathogen might be able to grow endophytically in plants from surviving infected seeds and negatively impact their growth. We successfully isolated the organism from cheatgrass leaf tissue after inoculating nondormant seeds, showing that that the organism can be an endophyte, and we obtained reductions in seedling growth from inoculated seeds for cheatgrass as well as at native grass species. *Festuca idahoensis* and *Bouteloua curtipendula* showed no difference in seedling biomass between infected and noninfected seedlings, whereas *Bromus tectorum* suffered a 10% reduction in growth and *Agropyron dasystachyum* growth was reduced 35%. This suggests that the pathogen could have a negative impact on native species that goes beyond simple seed mortality, a possibility that needs more thorough investigation.

We have also been examining the community ecology of the pathogen and both its native and annual brome hosts in more detail, using field experiments where we manipulate cheatgrass competition, water, and inoculum loads, and examine the effect on bluebunch wheatgrass and squirreltail emergence and establishment in precision seeding experiments at mesic and xeric sites (Merrill et al. in preparation).

We have also carried out extensive work on the population genetics of the cheatgrass host, including characterization of 96 Intermountain populations using our previously developed SSR markers for this species (Merrill et al. in review), as well as the development of over 100 SNP markers to help us better understand the role of outcrossing in this primarily inbreeding species. We are also working with variation in cheatgrass genetic traits that relate more directly to its interaction with *P. semeniperda*, including variation in resistance to the pathogen and in the tendency to form the secondarily dormant seed banks that are its primary target (Allen and Meyer 2010). In addition, we are using hydrothermal time experiments to unravel infection patterns observed in field retrieval experiments, which suggest that the pathogen may be able to infect under hydrothermal conditions that restrict seed germination (e.g., after summer thunderstorms), then sporulate rapidly when conditions become permissive (Allen et al. in preparation).

VI. Future Work Needed

There are three additional areas of research where we believe we could make rapid progress toward the goal of an effective biocontrol agent for annual bromes: (1) Based on the new knowledge that this organism is highly outcrossing and that hypervirulence is a relatively rare condition that is probably under negative selection in the wild, we will use

traditional breeding methods combined with marker-assisted selection to cross strains with desirable traits and screen the resulting progeny for biocontrol effectiveness; (2) To develop a protocol for producing and maintaining bulk inoculum of consistently high virulence, we will examine environmental and genetic factors contributing to variation in virulence expression among and within strains, using a combination of controlled-environment and gene expression studies; (3) Because the pilot inoculum production method we have developed is not suitable for large-scale application, we plan to carry out detailed optimization studies on production and delivery systems, followed by evaluation in scaled-up field inoculation trials. Ultimately we will need to partner with private industry to develop a commercial biocontrol product based on our research.

VII. Deliverables Crosswalk Table

Deliverable	**Description**	**Delivery Date**
Website	Online resource at www.bfod.org	Updated as needed
Technical meetings and tour presentations	Made 8 presentations for technical meetings and tours	10/2007- 5/2010
Society for Range Management national annual meeting presentations	Made presentations at 3 SRM national annual meetings	2/2009, 2/2010, 2/2011(accepted)
Other national meeting presentations	Made 16 additional presentations at national and international meetings	9/2007-6/2010
Master's thesis	M.S., Thomas Stewart, BYU	8/2008
Peer-reviewed publications	Promised 3, delivered 6: Beckstead *et al*, 2010, Beckstead *et al.* in press; Dooley and Beckstead 2010; Meyer *et al.* 2008, Meyer *et al.*, 2010, Stewart *et al.* 2009	12/2008-present
Final report	Summarized findings in a final report	9/30/2010

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

VIII. Publications and Presentations

Peer-reviewed Publications:

Beckstead J, Meyer SE, Connolly BM, Huck MB, and Street LE. 2010. Cheatgrass facilitates spillover of a seed bank pathogen onto native grass species. J. Ecology 98:168–177.

Dooley, SR and Beckstead, J. 2010. Characterizing the interaction between a fungal seed pathogen and a deleterious rhizobacteria for cheatgrass control. Biological Control 53: 197-203.

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