Final Report (June 1, 2010 - December 1, 2012)

Project Title: Developing a Delivery System for Annual Brome Biological Control Using the Seed Bank Pathogen Black Fingers of Death (*Pyrenophora semeniperda*)

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Introduction

The goal of our research program with the black fingers of death pathogen (*Pyrenophora semeniperda*) is to develop this organism as a mycoherbicidal biological control agent for invasive winter annual bromes in the context of revegetation/restoration seedings in the Intermountain West (IMW), including the Mojave Desert. This research targets both cheatgrass (*Bromus tectorum* L.) and red brome (*B. rubens* L.). This seed pathogen (BFOD) is ubiquitous in cheatgrass and red brome seed banks in the IMW. We are developing an augmentative or inundative biocontrol strategy for short-term knock-down or elimination of the brome carryover seed bank.

The principal objectives of the study were:

1) Develop a protocol for screening BFOD strains for superior performance (high virulence) in greenhouse bioassays using bulk inoculum, and screen strains that have shown promise in laboratory conidial inoculation trials.

2) Test BFOD strains selected for superior performance in field inoculation trials at cheatgrass and red brome sites.

3) Carry out experiments to optimize formulation/carrier technologies to increase effectiveness of annual brome biocontrol, evaluate alternative technologies using greenhouse bioassays, and test and compare the best technologies in field inoculation trials at cheatgrass and red brome sites.

4) Develop a bulk inoculum storage protocol for extending product shelf life.

Our progress on the first two years of this project was described in some detail in the first and second annual reports. We will summarize this earlier work and then describe in more detail the results of our field inoculation trials and also some more recent work on screening methodologies.

Summary of Progress June 2010-June 2012

Formulation/Carrier Technology Development

During the first year of this research project, we determined the conditions for optimal production of bulk inoculum on our original carrier, a calcined montmorillonite (Agsorb) product. These included drying the mixed bulk inoculum in a relatively thin layer under cool conditions (20C) following the addition of supplemental nutrient medium and a 24-hour sporulation induction treatment under visible and near-ultraviolet light. The next phase of our effort to optimize inoculum formulation focused on two components, namely finding an effective carrier with lower bulk density and lighter weight, and inventing a liquid inoculum/ supplemental medium formulation that would increase the density of infective units in the final product. Both of these improvements are needed to decrease the field application rate required to a level that is economically feasible and practical.

We have performed laboratory experiments with four potential carriers: calcined montmorillonite, vermiculite, perlite, and corncob grits. Evaluation of conidial densities per unit volume on these different carriers showed that vermiculite performed as well as calcined montmorillonite, while corn cob grits absorbed too much water and prevented drying at an optimal rate for sporulation at cool temperature, and perlite tended to dry out too quickly. We used vermiculite as the carrier in subsequent strain trials in the greenhouse and achieved seed mortality levels >90% at the relatively low inoculum load of 5 gram-equivalents per square foot. Because vermiculite has a bulk density about one quarter that of calcined clay, a gram-equivalent of vermiculite only weighs 0.25g, making it the carrier of choice so far in the quest for effective inoculum delivery with a light-weight carrier.

The other area where our formulation needed improvement was in the concentration of conidia or other infective units per unit volume of final product. We reasoned that, with a more concentrated inoculum, we should be able to further reduce the application rate. To achieve this, we have experimented with different liquid culture media, different initial inoculum loads in liquid culture, and different amounts and types of supplemental media. We were able to demonstrate that use of MAM (modified alphacel medium, which contains primarily oatmeal and coconut milk) as a supplemental medium significantly increased conidial concentrations in the final product relative to both PDB (potato dextrose broth) and V8 broth, when the fungus was grown in PDB as a culture medium.

Culturing the fungus directly in a liquid MAM medium resulted in low mycelial yield, which contradicted results of an experiment reported earlier. V8 broth did increase mycelial yield relative to PDB. More recently we have tried another liquid medium, TO medium (main ingredients tomato paste and oat flour), and this has given us not only the highest mycelial yields in liquid culture but also substantially better sporulation when used as a supplemental medium. For our most recent field inoculation experiments (installed in fall 2012 with Joint Fire Sciences Program funding), we used bulk inoculum prepared with TO as the medium and with vermiculite as a carrier.

Bulk Inoculum Storage Protocols

As reported earlier, we have the results of yearly testing for four years using bulk inoculum produced in summer 2008 for our original field trials and stored at several

temperatures from -20C to +30C. We have tested this bulk inoculum by applying it directly to dormant cheatgrass seeds in petri dishes at 20C and measuring its ability to cause disease. To date there has been no decline in the ability to cause disease using this method of determination, at any storage temperature. This indicates that the bulk inoculum is extremely stable and will have a long shelf life, a definite asset for a commercial biocontrol product.

Pathogen Strain Screening

We decided to examine ten new pathogen strains in some detail, in an effort to identify traits that would enable us to predict field biocontrol efficacy. The ten strains were selected from a larger set of 100 strains collected in 2010 from three field populations in northern Utah where virulent strains had been isolated in earlier studies; Tenmile Creek, Boxelder County, Utah (TMC), the Whiterocks Study Exclosure in Skull Valley, Tooele County, Utah (WRK), and a site a few miles further west on the Whiterocks Road (WRR). The ten intensively studied strains were selected to represent a range of mycelial growth rates (MGI's) based on a growth rate screening of the 100-strain collection. There was a four-fold difference in MGI values among the selected strains. Our earlier work had suggested that MGI might be negatively associated with at least one form of virulence, i.e., faster growing strains were less virulent on nondormant cheatgrass seeds than slower growing strains (Meyer et al. 2010).

Traits included in previous pathogen screening trials have included host seed mortality at low conidial inoculum loads on dormant seeds, host seed mortality at high conidial inoculum loads on nondormant seeds, and dormant host seed mortality in ring microcosms with autoclaved seed zone samples and laboratory produced bulk inoculum, as well as phytotoxin production in both liquid and solid culture. We have had considerable difficulty developing repeatable screening methodologies, but have now overcome some of these problems and will report results of more reliable screening trials below.

We obtained considerable evidence in earlier field trials that this pathogen can sometimes kill a sizeable fraction of the part of the seed bank that would otherwise germinate in the first rainfall event, i.e., nondormant seeds. We developed the hypothesis that the mechanism for this successful attack on nondormant seeds might be associated with infection at water potentials too low to permit seed germination. Our graduate student Heather Finch, funded partially through this project, carried out a series of laboratory experiments to test this idea (Finch et al. 2013). Our approach in the first experiment was to inoculate both dormant and nondormant seeds at relatively low levels (1:100 conidia:talc by weight), incubate them at a series of water potentials from -0.5 t0 -2.0 MPa for four weeks, then transfer the seeds to free water and score seed germination and pathogen-caused seed mortality.

All nondormant seeds escaped mortality in the 0 MPa (directly into free water) treatment and the -0.5 MPa treatment, because the seeds could germinate so rapidly under these conditions that they escaped mortality. At -1.0 MPa, only about 15% of the seeds were killed, because even at this water potential a majority of the seeds could germinate quickly enough to escape. At -1.5 and -2.0 MPa, a majority of the nondormant seeds were killed, and the rapid appearance of fruiting bodies upon transfer to water showed that these seeds were infected and probably already killed in the low water potential treatments that suppressed germination of nondormant seeds.

This experiment supports the idea of the pathogen gaining the advantage in the 'race for survival' at low water potentials that suppress germination of nondormant seeds, and could explain how it is able to kill nondormant seeds in field seed banks. Follow-up studies have shown that this effect can also be observed across a range of temperatures and also under regimes more like natural conditions in the field, where seeds experience full hydration followed by periods of more or less rapid and complete dehydration. This suggests the possibility of screening pathogen strains for their ability to operate optimally under low and fluctuating water potential conditions, as another possible tool for the selection of maximally effective strains for biocontrol. These strain trials include three components: conidial germination, mycelial growth rate, and ability to cause nondormant seed mortality, each as a function of both temperature and water potential. The results of these experiments are reported below.

Field Inoculation Trials 2011-2012

The field inoculation trials had two main objectives. First, we wanted to determine whether our improved formulation with the lighter weight vermiculite carrier and the MAM supplemental medium would give us better control at low inoculum loads than the original formulation. Second, we wanted to relate our laboratory strain trial results to results with selected strains from these trials in the field.

We installed the field inoculation trials at two sites in late August, 2011, prior to any germination triggering rainfall. These included a cheatgrass site at the Whiterocks Exclosure in Skull Valley, Tooele County, Utah, and a red brome site in Pakoon Basin in the Grand Canyon-Parashant National Monument in northwestern Arizona. Part of the red brome site burned during the planning of this experiment (Hidden Fire), enabling us to include burned and unburned treatments in the design for that site. At the Whiterocks site, only the unburned treatment was included. Designs for the two sites were otherwise parallel. Main effects (other than burn at Pakoon Basin) were inoculum formulation, inoculum load, and strain. Three inoculum formulations were included: the original formulation with the calcined montmorillonite carrier and PDB as a supplemental medium, calcined montmorillonite clay with MAM as the supplemental medium, and vermiculite with MAM as the supplemental medium. This permitted us to determine whether vermiculite performed as well as calcined montmorillonite on a volume basis, and to determine whether MAM performed better than PDB as a supplemental medium on vermiculite. All inoculum was produced from mycelium produced in liquid PDB culture, using the optimized production protocols we had developed
. We deliberately selected inoculum loads not necessarily expected to give complete control in the field, because our primary goals were to compare treatments and to find out whether we could get improved control at lower application rates. We therefore applied inoculum at the rate of 5 and 15g-equivalents per square foot plot, rather than the 45 g-equivalents that gave us complete or near-complete control in earlier experiments.

We picked the four best new strains based on our laboratory data to include in the field inoculation trials: TMC1022, WRK1022, WRR1016, and WRR1029. We also included two of the original strains used in field trials at these two sites, for comparison purposes: TMC23 and DOG3. Lastly, we included a treatment that was a mixture of two strains (WRR1016 and WRR1029). The mixture treatment was intended to determine whether

combining strains would result in a positive synergistic effect, a competitive effect, or a simple additive effect. We also included an uninoculated control treatment.

The original inoculum formulation was included in the factorial design only with the strains used in the earlier trials for a sub-experiment of 10 blocks x 2 strains x 2 levels x 3 inoculum formulations = 120 plots + 10 control plots=130 plots total. The two new formulations were factorially combined in a sub-experiment with the remaining strains: 10 blocks x 5 strains x 2 levels x 2 formulations = 200 plots. This sub-experiment used the same control plots, for a total of 330 plots at the Whiterocks exclosure. The same design was used on the unburned treatment at Pakoon, but only the first half of the design, with the original strains and the three inoculum formulations, was installed on the burned treatment, for a total of 330 + 130 =460 plots.

We added supplemental seeds to each plot at the time of inoculation to reduce plot to plot variability in seed density: 1000 cheatgrass seeds per plot were added at the Whiterocks site and 250 red brome seeds per plot at the Pakoon site. The lower number at Pakoon was dictated by lack of available seeds.

Seed bank samples were obtained from each of the 460 plots at Pakoon in early April and from each of the 330 plots at Whiterocks in early May, prior to dispersal of current-year seeds into the plots. Samples were taken with soil cans 6 cm in diameter and 4.5 cm deep, from the center of each plot. The samples were dry when collected, and were placed in individual labeled paper sacks for storage. The samples were stored dry in a warm greenhouse breezeway for several weeks to permit any viable seeds in the sample to lose dormancy prior to processing. This makes it much easier to determine which seeds remaining in the sample are still viable.

New Results – June 2012-December 2012

Field Inoculation Trials

We obtained strongly contrasting results at the two sites where field inoculation trials were installed. At the Whiterocks Exclosure, in Skull Valley, Utah, approximately 100 miles west of Provo, Utah, background levels of disease were very high, with over 18,000 killed seeds–m^{-2}, and the killed seed proportion (killed seeds / (killed + viable seeds) was also high in the uninoculated controls (78%). There were no significant treatment effects in the inoculation experiment at Whiterocks, regardless of formulation, strain, or inoculum load (Figure 1). This was different from results from earlier years, in which inoculum addition at these relatively low loads resulted in a significant increase in killed seed proportion. We obtained no treatment effect of inoculum addition even at a high load (45 ge/ft^{-2}) in another experiment at Whiterocks in 2009-2010 (Meyer et al. in press). Possible reasons for the ineffectiveness of inoculum treatments at Whiterocks are discussed in the 'Future Research' section below.

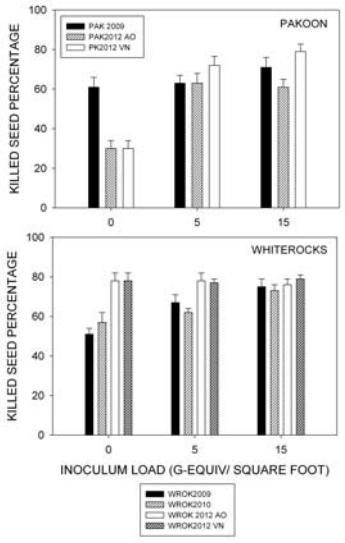

Figure 1.

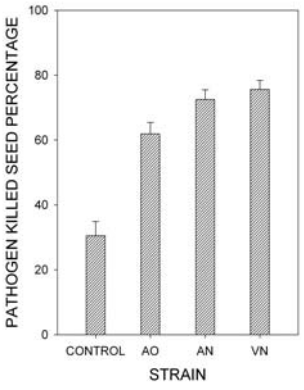

Figure 2.

At the Pakoon study site, where the principal annual grass present was red brome (*B. rubens*), we observed marked treatment effects. In the factorial that included three formulations, two inoculum levels (plus an uninoculated control), and two strains, there was a highly significant effect of formulation on killed seed percentage (SAS Proc Mixed; F=5.71; P=0.0039). Both formulations that used MAM as the supplemental medium resulted in a 15% increase in killed seed proportion relative to original Agsorb clay carrier with PDB as the supplemental medium (AO), and there was a trend for the vermiculite carrier (VN) to outperform the Agsorb carrier (AN) even on a gram-equivalent basis. When the lower bulk density of vermiculite is taken into account, the use of vermiculite with MAM resulted in a five-fold decrease in the application rate by weight relative to the original formulation for an equivalent level of control. All three formulations resulted in a major increase in pathogen-killed seed percentage relative to the uninoculated control. The increased effectiveness of the VN formulation over the AO formulation was especially apparent at the 15 ge-ft^{-2} application rate (Figure 1; formulation by inoculum load interaction: F=4.26; P=0.0398).

There were significant differences in killed seed proportion among strains at Pakoon, but these differences were generally not very large, and depended in part on the formulation used (strain main effect: F=2.88, P=0.0097; strain by formulation interaction: F=2.42, P=0.0266; Figure 3). All strains had mean killed seed proportions significantly higher than the control. The mixed strain treatment resulted in a mean killed seed proportion significantly lower than that of any pure strain, indicating that in this case the interaction between strains was antagonistic. Only TMC23, an effective biocontrol strain used in earlier trials, was strongly affected by formulation. It killed an average of 25% more seeds when applied in the vermiculite carrier.

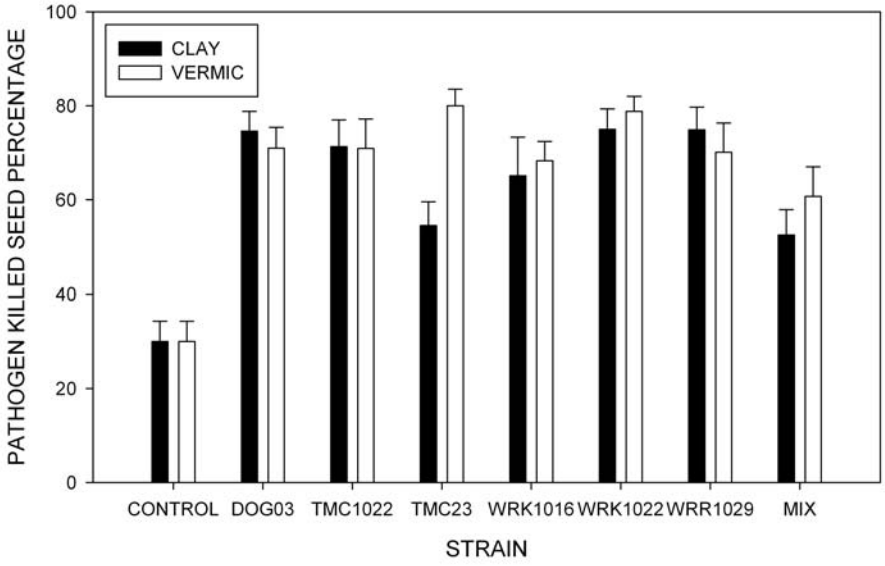

Figure 3.

There may still be room for improvement in the development of inoculum formulations that are effective as delivery systems for this biocontrol organism, but even with a five-fold decrease in the weight of inoculum shown to be effective, we are still dealing with a massive amount of inoculum in order to achieve useful levels of biocontrol. Our best hope for lowering the application rate for effective control lies in the discovery of more virulent and/or more environmentally tolerant strains, and therefore in developing better methodologies for screening, selection, and maintenance of suitable strains.

Screening and Selection

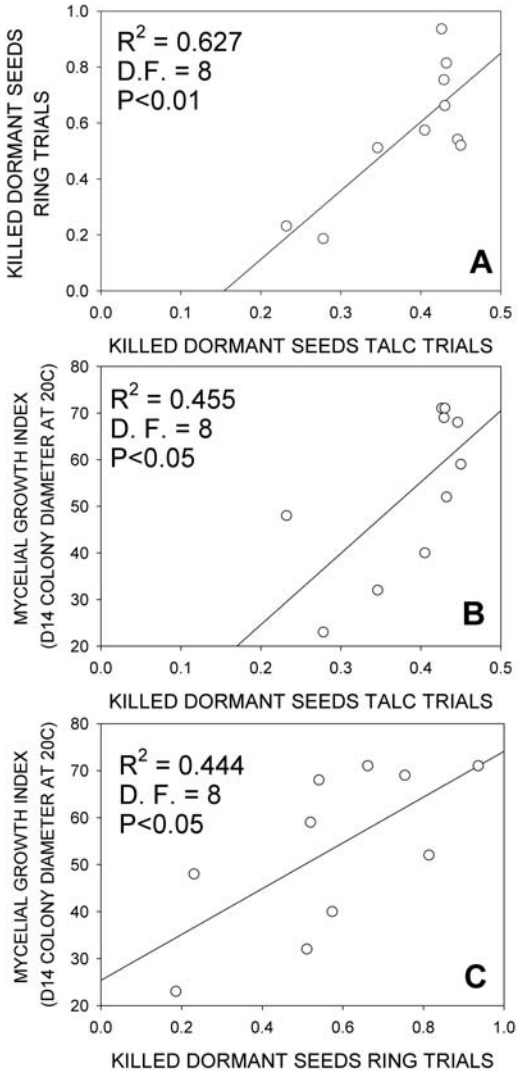

Figure 4.

Our virulence screening methods have been plagued with lack of repeatability, but we have finally developed a technique for screening for virulence at low inoculum loads (diluted in talc) on dormant seeds that gives consistent results. Virulence data for ten strains developed using these improved methods are reasonably well-correlated both with both mycelial growth rate and with seed mortality in ring microcosm trials (Figure 4). These results show *ex post facto* that the strains used in the field trials all had similarly high levels of virulence as measured in conidial inoculation (talc) trials, even though their performance in ring microcosms varied quite widely. The conidial inoculation data set, which showed these strains to be quite similar in virulence, proved to be a better predictor of performance in the field.

The use of bulk inoculum in a screening procedure introduces many sources of variation that are not seen in a conidial inoculation trial. The virulence of the strain interacts with the quality of the inoculum in terms of conidial density, and this can vary by batch due to limited ability to control production conditions. Environmental conditions in the seed bed microcosms are more similar to field conditions but also far more variable than environmental conditions in a petri dish.

One interesting outcome of the these correlation analyses is that in the case of virulence on dormant seeds, strains with a faster growth rate are generally also more virulent. This trend is opposite to the trend we observed with respect to growth rate and virulence on nondormant seeds (Meyer et al. 2010). In nondormant seed inoculations at high inoculum loads, there is a negative correlation between mycelial growth rate and virulence—slower growing strains are better able to kill nondormant seeds. We

hypothesized that this pattern might be due to the necessity of producing metabolically expensive secondary metabolites to cripple germinating seeds, and cytochalasin B, which is produced in large amounts by this fungus, seems a likely candidate phytotoxin (Evidente et al. 2002). In any case, the fact that faster strains kill more dormant seeds and slower strains kill more nondormant seeds suggests an evolutionary mechanism for the maintenance of genetic variation in growth rate in this pathogen.

We have completed a set of studies exploring the basis of the ability of this pathogen to kill nondormant seeds in the seed bank under conditions of water stress. Our earlier work demonstrated that rapidly germinating nondormant seeds that would normally escape the pathogen can suffer high mortality if inoculated seeds are incubated under germination suppressive conditions at low water potential (Finch et al. 2013). We have now directly examined the ability of the pathogen to undergo conidial germination and growth at reduced water potentials in an effort to understand the mechanism by which seed mortality can take place under these conditions, and we have also begun to screen a panel of strains to look for variation in the ability of the pathogen to function under these conditions. If we could find a strain with extraordinary ability to germinate, grow, and infect under water stress, it could be a very useful step forward for biocontrol. Such a strain might be able to kill a high proportion of nondormant seeds. To examine this possibility, we first measured mycelial growth rate as a function of water potential at two temperatures for 11 strains of the fungus (Figure 5).

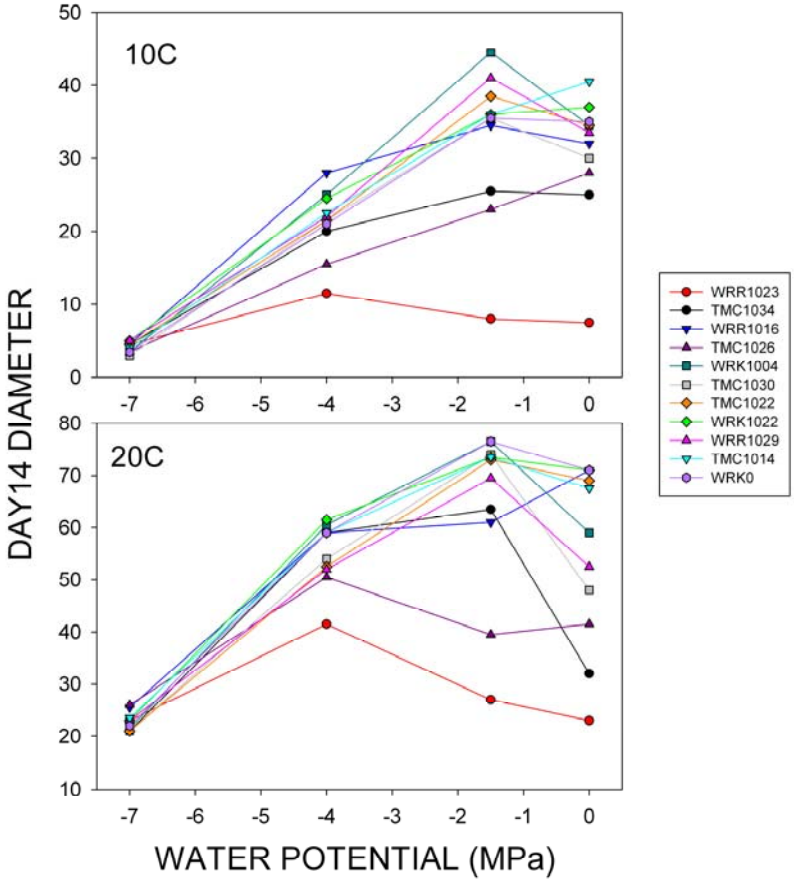

Figure 5.

There are several striking things about Figure 5. First, it is clear that most strains of the pathogen are capable of relatively rapid growth at water potentials as low as -4 MPa (-40 bars), a water potential far below that at which most seeds can germinate or exhibit seedling growth. Second, particularly at 20C, most of the pathogen strains, especially the faster-growing strains, have an optimal water potential for growth that is below 0 MPa. This fungus appears to grow optimally at -1.5 MPa, which is equivalent to the permanent wilting point for plants. Third, the slower growing strains actually appear to have even lower optimal water potentials for growth than the fast growing strains (e.g., WRR1023, orange circles). And last, there is indeed considerable variability among strains in the ability to continue rapid growth at relatively low water potentials, potentially providing a substrate for selection for a more water stress tolerant strain.

Another important aspect of the ability of the pathogen to function at low water potential is conidial germination. We only have provisional data on this process, but all indications are that conidial germination can also take place at remarkably low water potentials (Figure 6).

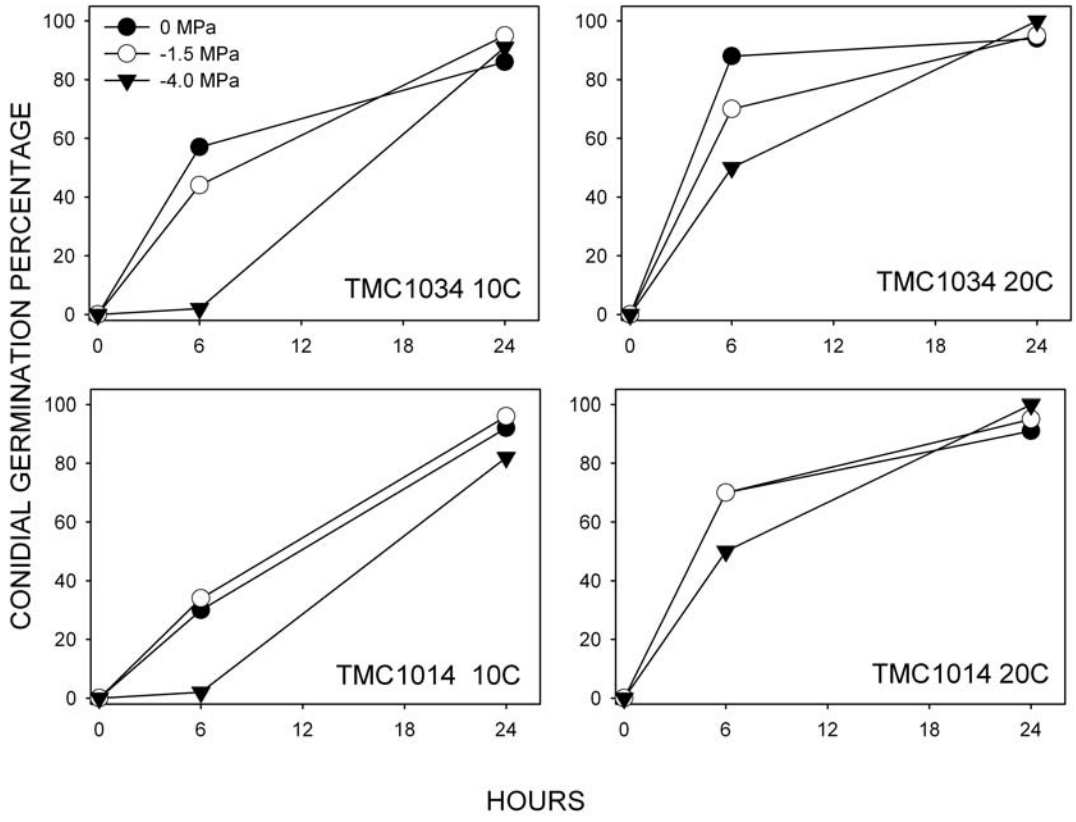

Figure 6.

For two pathogen strains, we measured time courses of conidial germination at two temperatures and three water potentials. Even at 10C and -4 MPa, germination is essentially complete by 24 hours, and under favorable conditions can be largely complete by 6 hours. These tests were carried out on water agar. On a nutrient medium such as PDA, germination is even faster. In general there is very little decrease in germination speed from 0 to -1.5

MPa, a result in accord with the results for mycelial growth rate. We do not yet know how much among-strain variation there is for conidial germination traits. Conidial germination studies are complicated by the fact that lots of conidia from the same strain produced at different times under slightly different conditions may exhibit variation both in quality and in initial germinability (dormancy). It will require more detailed studies to understand how conidial germination and its variability will impact the ability of a strain to function as an effective biocontrol agent under water stress.

We have also examined among-strain variation in the ability to kill seeds under water stress in a system similar to that described in Finch et al. (2013). Inoculated seeds (1:100 conidia:talc) were incubated at -2.0 MPa on polyethylene glycol-saturated blotters in petri dishes for 4 wks at 10 or 20C, then transferred to water. Fungal stromata that protrude from the seeds within a week of transfer to water have resulted from infection that took place in the low water potential treatment. Strains varied considerably in their ability to carry out this

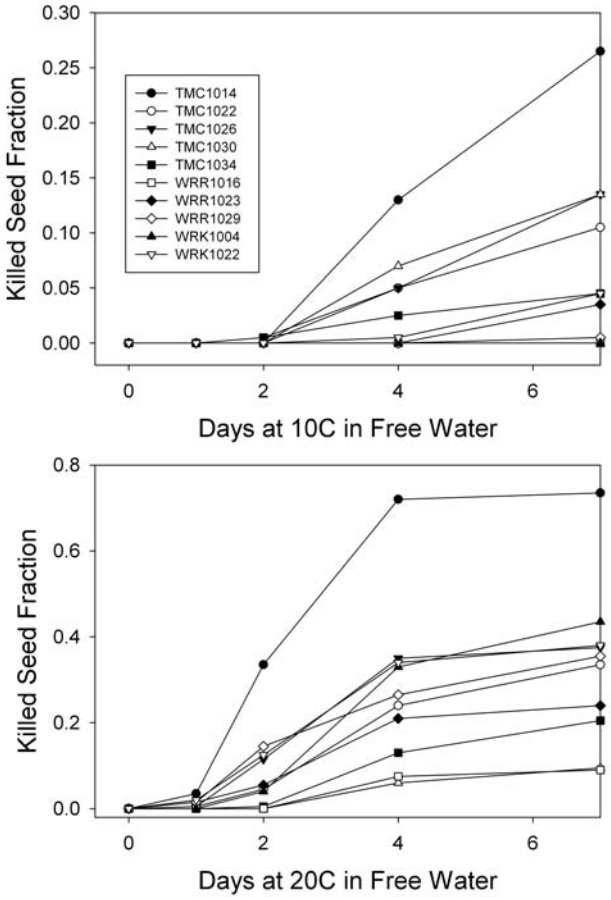

Figure 7.

infection process (Figure 7). The most effective strain in the trial was TMC1014, which was also one of the faster, more virulent strains in other tests. It was more effective than the WRK0 strain used in Finch et al. (2013), which caused 59% mortality 7 days after the switch from -2.0 MPa at 20C.

Conclusions and Future Research

This research project has resulted in several knowledge outcomes that both encourage us that this pathogen has potential for biocontrol and alert us to the pitfalls of this kind of research and development project. On the positive side, we have learned that bulk inoculum produced in a system with potential for industrial scale-up can be effective in reducing brome persistent seed banks under field conditions, and we have advanced development of a delivery system for this biocontrol to the point where we can reduce application rates by a factor of five and still obtain complete control under some scenarios. We have determined that bulk inoculum prepared according to our protocols has a much longer shelf life as a dry, granular formulation than is the norm for biocontrol organisms. We have determined that the pathogen exhibits genetic variation for many traits with implications for biocontrol, including virulence on dormant vs. nondormant seeds, mycelial growth rate, phytotoxin production, and tolerance to non-optimal temperatures and water potentials. We have devised methods for measuring this variation and for testing predictions about its effects in the laboratory and greenhouse and also in the field. We have taken these knowledge outcomes to the next step: application for a US patent for our technology. This patent application through the US Forest Service is currently pending.

On the minus side, we have found that there are populations of annual bromes where application of even high inoculum loads does not result in complete elimination of the carryover seed bank or even sometimes in decreases in carryover seed density. The method works best at sites where natural pathogen levels are generally lower, which suggests that perhaps consistently high pathogen loads result in selection for more resistant host genotypes. This selection would be even stronger at a place like Whiterocks, where periodic stand failure (‘die-off’, probably due to other soilborne pathogens; Baughman and Meyer 2013) is a regular occurrence. Because the host population must recover from the carryover seed bank after a die-off, these events exert strong selection pressure for resistance to black fingers of death, as only seeds that escape mortality in the carryover seed bank are present to establish a stand the following year. We are currently in the process of establishing experimental work to determine whether selection for disease resistance in cheatgrass is driven by high pathogen loads and die-off events.

In a non-race-specific pathosystem like this one, the only biocontrol remedy for increased general resistance is increased general virulence, making it even more imperative to understand the range of variation in virulence and the genetic factors that contribute to its expression in this pathogen. It is possible that there are fitness trade-offs for excessive virulence, so that strains that evolve to be hypervirulent do not persist in natural populations. The art for biocontrol is to discover and increase these strains, deploy them for short term biocontrol, and then allow the process of natural selection to remove them from the system. For this reason our emphasis for the next phase of the research will be on developing a better mechanistic understanding of different kinds of virulence in this pathogen and their fitness consequences, and on developing more sophisticated methods for screening a much larger number of isolates to identify hypervirulent strains that may prove to be quite rare.

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