

**THE GRASS SEED PATHOGEN *PYRENOPHORA SEMENIPERDA* AS A
BIOCONTROL AGENT FOR ANNUAL BROME GRASSES**

By

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ABSTRACT

THE GRASS SEED PATHOGEN *PYRENOPHORA SEMENIPERDA* AS A BIOCONTROL AGENT FOR ANNUAL BROME GRASSES

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Master of Science

Bromus tectorum and other annual brome grasses have invaded many ecosystems of the western United States, and because of an annual-grass influenced alteration of the natural fire cycle on arid western range lands near monocultures are created and conditions in which the native vegetation cannot compete are established. Each year thousands of hectares become near monocultures of annual brome grasses. *Pyrenophora semeniperda*, a generalist seed pathogen of annual grasses, shows major potential as a possible mycoherbicide that could help in reducing the monocultures created by annual grasses. The purpose of this research was to identify the requirements for isolating cultures of *P. semeniperda*, search for a hypervirulent strain, and evaluate its effect in the field. The techniques for isolating the fungus have evolved and become more efficient. The first two years of working with *P. semeniperda* resulted in 11 isolates. During the third year of this study, we developed a single spore isolation technique that resulted in 480 additional isolates. Virulence screening resulted in detection of a range of isolate ability to kill non-dormant *B. tectorum* seeds. Ninety-two isolates represented a range of

virulence from 0-44%. The variation in virulence was expressed mostly within populations rather than between populations. Similarly, virulence varied significantly within Internal Transcribed Spacer (ITS) genotypes and habitats but not between them. When conidial inoculum was applied in the field there was no observed difference in disease incidence between different levels of inoculum. This is thought to have been due to applying the inoculum under conditions in which most *in situ* seeds were infected and killed by already high field inoculum loads. While additional field trials are needed to optimize the inoculum effectiveness, the overall results of this research provide a good foundation for using *P. semeniperda* as a biological control for seed banks of annual brome grasses.

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TABLE OF CONTENTS

Graduate Committee Approval	ii
Final Reading Approval and Acceptance	iii
Abstract	iv
Literature Review.....	1
Introduction.....	1
<i>Pyrenophora semeniperda</i> Background Information	2
Controlling Cheatgrass with <i>Pyrenophora semeniperda</i>	3
Research Hypotheses	6
Literature Cited	8
Discovery of <i>Pyrenophora semeniperda</i> in the Old World	10
Abstract	10
Technical Note	10
Literature Cited	14
As Accepted for Publication	15
Figures for Discovery of <i>Pyrenophora semeniperda</i> in the Old World	17
Virulence Variation in the Seed Bank Pathogen <i>Pyrenophora semeniperda</i>	18
Abstract	18
Introduction.....	19
Materials and Methods.....	23
Isolation and Conidial Production.	23
Conidial Viability Evaluation.	24
Initial Virulence Screening.	25
Repeated Virulence Experiment.	25
Mycelial Growth Rate Studies.	26
Results.....	27
Conidial Viability Evaluation.	27
Initial Virulence Screening.	27
Repeated Virulence Experiment.	28
Mycelial Growth Rate Studies.	29
Discussion.....	29
Acknowledgements.....	34
Literature Cited	35
Table 1.	39
Table 2.	40
Table 3.	41
Fig. 1.	42
Fig. 2.	43
Fig. 3.	44
Fig. 4.	45
Appendix A.....	46

Isolation Method: Whole Stromata Isolation	46
Appendix B	49
Isolation Method: Single Conidia	49
Appendix C	52
Isolation Methods Data	52
Experiment 1	52
Fig. 1.	54
Fig. 2.	55
Experiment 2	56
Fig. 1.	58
Fig. 2.	59
Appendix D	60
Agar Recipe: Modified Alphacel Medium	60
Agar Recipe: V8 Juice Agar	60
Appendix E	61
Field Trail Data	61

Literature Review

Introduction

Wildland fire in the western United States is becoming a more and more prominent problem that is costing taxpayers billions of dollars annually. One major reason for this increasing concern is the impact created by the invasive annual bromes, cheatgrass (*Bromus tectorum*) and red brome (*Bromus rubens*). Annual bromes are able to alter natural fire conditions. Annual bromes have a “winter annual” life cycle that differs from that of the perennial native grasses. Seeds germinate in fall or early winter so that established plants grow rapidly in early spring when moisture is most available. This growth habit provides a competitive advantage over many of the native species.

Abundant seeds are produced and the plant’s life cycle is completed in early summer, while native bunchgrasses are still green and not yet reproductively mature. Dry, dead brome litter creates an increase in fuel loads and fills the interspace between woody species and bunchgrasses. This early dry fuel burns readily and produces a continuous layer of fuel to carry range fires. The resulting fires put native vegetation at a disadvantage because many species have not yet produced mature seed. A positive feedback loop is created, and with each successive fire cheatgrass becomes more dominant and the fire interval shortens. This reduced fire cycle creates obstacles for seeding as part of post-burn rehabilitation above the normal obstacles an arid-ecosystem seeding already has.

A major obstacle to seeding into landscapes invaded by annual bromes with native seeds after a fire is the increased competition from annual brome grasses. While wildfires deplete the annual brome seed bank, many viable seeds remain. Conventionally, seeding takes place as quickly as possible after a fire. This is to try to get the seedlings

established before brome competition builds up. However, in an arid ecosystem these seedlings often fail due to lack of precipitation, and the window to beat brome competition quickly closes. Over time, invaded sites can become entirely dominated by annual grasses, producing a near monoculture. In this condition wildfires no longer burn hot enough to destroy many of the seeds in the seed bank. If we could find a way to remove the residual annual brome seed bank following wildfire, the probability of successful rehabilitation of annual brome monocultures would greatly increase.

***Pyrenophora semeniperda* Background Information**

Pyrenophora semeniperda (Brittlebank and Adam) Shoemaker (anamorph *Drechslera campanulata* (Lev.) Sutton) (black fingers of death) has been considered for use as a biocontrol of annual grass weeds in cereal crops (Medd et al 2003; Medd and Campbell 2005). It is most commonly found infecting cool season grasses and occasionally the seeds of broad leaf species. In most scientific literature *P. semeniperda* is described as a weak pathogen that rarely causes seed mortality and has minimal impact on seedling growth (Medd et al. 2003a). It has been observed that seed death is a common consequence of infection. Beckstead et al. (2007) described the infection process as a race for the endosperm reserves of the seed. A major determinant of seed death was germination rate; seeds that germinated quickly escaped death while those that slowly germinated succumbed to the fungus.

Germination rate is influenced by the dormancy status of the seed (Allen and Meyer 2002). If the fall is dry and insufficient precipitation falls to trigger a germination event, then most seeds enter secondary dormancy. A few remain non-dormant and are able to germinate quickly. Dormant seeds are the primary food source for *P.*

semeniperda. This is evidenced by killed seed densities as high as 50,000 per square meter in arid sites where high levels of secondary seed dormancy are common (S. Meyer, personal communication, 2009). This leads us to believe that *P. semeniperda* could be a major seed pathogen.

Isolates of *P. semeniperda* vary in virulence, a characteristic that is important in being able to control annual bromes. Virulence variation in this study is as variation in the pathogen's ability to kill non-dormant cheatgrass seeds. *Pyrenophora semeniperda* is a sexually reproducing organism, and it is likely that through recombination new virulence strains are being produced. Efforts to screen a large set of isolates could help identify a highly virulent strain of the fungus, which would aid in efforts to use *P. semeniperda* as a biological control for invasive annual bromes.

Controlling Cheatgrass with *Pyrenophora semeniperda*

Bromus tectorum L. (cheatgrass) is an invasive annual Eurasian grass. It is one of the main plants responsible for an increase in frequency and intensity of fires on western wildlands. The 2006 reported cost of wildfires to U.S. agencies was over 1.5 billion dollars (Cohan and Burnett 2008). These fires destroy native plant communities and reduce biodiversity. Due to near monocultures of the highly flammable *B. tectorum*, major fires now occur every three to five years instead of every 60-100 years (Whisenant 1990). The native plant communities affected by this change in fire frequency are often unable to recover and in many areas are destroyed. Over 41 million acres in North America have been invaded with significant loss of native vegetation (Whisenant 1990).

Land managers face a critical need to control weedy annual grasses (Gallandt 2006). The seed stage of an annual is very difficult to target; however, seed mortality is

very important to population dynamics of a species. Gallandt (2006) further pointed out the importance in targeting the seed bank to lower plant densities. Chee-Sanford et al. (2006) echoed Gallandt's call for targeting the seed bank, also stressing that microorganisms play a big role in seed bank dynamics.

Annual bromes can be controlled through three different approaches: physical, chemical, or biological control. The key is to create a disturbance in the life cycle of the specific weed targeted. Physical control involves the use of a method such as plowing, mowing, fire, etc., to achieve a disruption to the life cycle. Chemical control utilizes herbicides to reduce growth or kill plants (Duval 1997). Biocontrol involves the deliberate use of natural enemies to reduce the density of a weed to tolerable levels or to achieve complete eradication (Watson 1998). These methods can be used independently or in any combination to disturb the life cycles of target species. With annual bromes a combination of these control methods will most likely be needed.

Current control methods for annual brome grasses are limited and each has disadvantages. Burning can eliminate most seed production when done early in the season, but this may not affect seeds already present in the soil seed bank. Due to the dry conditions and fine fuel produced by *B. tectorum*, controlled burns can result in rapid and intense burning conditions that are unpredictable and carry the risk of escaping containment. Tillage is expensive to undertake on the scale needed to control annual bromes and possesses the risk of damaging remnant native vegetation. Herbicides can be effective as a control measure, but are expensive and may adversely affect non-target species as well.

An aggressive, introduced weed that infests large areas is an ideal candidate for use of a biocontrol agent. Annual bromes such as *B. tectorum* and *B. rubens* fit this definition perfectly. They readily invade disturbed areas and cover millions of hectares in the United States.

One other microorganism has been researched as a biocontrol agent for annual bromes. *Pseudomonas fluorescens*, a root colonizing bacterium, inhibits brome growth especially in agar plate bioassays (Kennedy et al. 2001). These bioassays were performed on seven brome species with root inhibition and plant growth reduction averaging 87%.

Pyrenophora semeniperda, a pathogen of grass seeds, has the potential to reduce the field seed bank of *B. tectorum*. Using *P. semeniperda* as a biocontrol on *B. tectorum* could improve restoration efforts as well as help reduce the risk of fire to western wildlands.

Pyrenophora semeniperda is a generalist pathogen that is known to attack *B. tectorum* seeds and other annual bromes (Beckstead et al. 2007). Infection is evident from the development of macroscopic fungal stromata on the seed (Meyer et al. 2007). *Pyrenophora semeniperda* is better able to infect and kill slow-germinating or dormant seeds, but quick-germinating seeds usually escape death. This is due to competition for the endosperm resources between the germinating seed and *P. semeniperda* (Beckstead et al. 2007). Under field conditions the primary targets of *P. semeniperda* are secondarily dormant seeds in the spring seed bank.

Medd and Campbell (2005) studied grass seed infection by *P. semeniperda* and the possibility of its use as a biocontrol for weedy species. They inoculated developing

seeds in the inflorescence, and found that an inoculum of conidial suspension resulted in greater infection than an inoculum of mycelium fragments. In the field they had infection as high as 70%.

Isolates of *P. semeniperda* are known to vary in virulence, and the degree of virulence appears to be related to the levels of production of toxic metabolites (Campbell et al. 2003a). In previous studies virulence was measured using leaf spot and wheat seedling bioassays, not by the ability of the fungus to kill non-dormant seeds.

Campbell et al. (2003b) also researched ideal conditions for laboratory growth of *P. semeniperda*. They found that maximum growth of *P. semeniperda* in culture required an alternating light/dark cycle, with incubation at 23C during the light phase and 19C during the dark phase. Modified alphacel medium (MAM) was the optimal medium for culturing the fungus. Under these optimal conditions an increase of conidial numbers of 800% was observed. Previous studies suggest potential for the use of *P. semeniperda* as a biological control for *B. tectorum* as well as other invasive annual brome grasses.

Research Hypotheses

The goals of this thesis research were 1) to learn how to obtain black fingers of death in culture, 2) to learn whether different isolates of black fingers of death vary in their ability to kill non-dormant host seeds, and 3) to learn whether mortality of host carryover seeds in the field can be increased/decreased by manipulating inoculum load.

Specific hypotheses include:

1. The production of conidia from pure isolates of BFOD can be optimized by manipulating cultural conditions and methodologies, including temperature, light, and sterilization technique.

2. A) The black fingers of death (BFOD) isolates will exhibit different degrees of virulence, as measured by the ability of conidial inoculum to kill non-dormant, fast germinating cheatgrass seeds. B) The degree of virulence of a BFOD isolate will be positively correlated with its growth rate in pure culture.
3. Host carryover seed survival in the field will be inversely correlated with BFOD inoculum loads.

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Discovery of *Pyrenophora semeniperda* in the Old World

Accepted (pending revision) by Plant Disease

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Abstract

Pyrenophora semeniperda's current reported distribution worldwide is mainly temperate grasslands and winter cereal growing regions. Previous reports in Europe and Asia were not known, and strong doubt has been expressed that it occurs in the Old World. However, it has been discovered in Turkey and Greece. Using Internal Transcribed Spacer (ITS) for genetic identification, *P. semeniperda*'s existence in both Europe and Asia has been confirmed.

Technical Note

Pyrenophora semeniperda (Brittlebank and Adam) Shoemaker (anamorph *Drechslera campanulata* (Lev.) Sutton) is a generalist plant pathogen that can cause leaf spot disease and seed rot of annual and perennial grasses. It has also been found to infect several dicotyledonous species. *Pyrenophora semeniperda* is a weak pathogen when it infects the leaves or stems of grasses (Medd and Jones 1992), but shows potential to be a major pathogen of seeds. Evidence of seed infection is easily observed from the development of macroscopic fungal stromata on the seed (Meyer et al. 2007) (Fig. 1). We have observed that seed mortality due to *P. semeniperda* is inversely correlated with

germination rate, i.e. slow germinating seeds are more susceptible while fast germinating seeds usually escape death. This is due to competition for the endosperm resources between the germinating embryo and *P. semeniperda* (Beckstead et al. 2007). Under field conditions the primary targets of *P. semeniperda* are dormant seeds which fail to germinate or germinate very slowly when wetted. We are currently studying *P. semeniperda* as a possible bio-herbicide for use as a pre-emergent seed control of annual grasses.

Pyrenophora semeniperda has been reported in Argentina, Australia, Canada, Egypt, New Zealand, South Africa, and the United States (IMI 1995). It was originally described in France in the mid 1800's (Leveille 1841), but it has not been reported in Europe since. Past reports in Asia are not known, and Medd and Jones (1992) expressed strong doubt that the fungus occurs naturally in the Old World.

In May 2008, we observed what appeared to be *P. semeniperda* in Asia. The characteristic fungal stromata were observed in Pamukkale, Turkey (37° 54'32.37"N 29° 07'44.51"E) on a *Taeniatherum caput-medusae* (L.) Nevski (medusa head) seed. Pamukkale is located in the south-west province of Denizil. On six *Bromus tectorum* seeds fungal stromata were observed and collected in the Cappadocia region of Turkey (38° 39'33.59"N 34° 49' 16.54"E), in Love Valley outside the city of Goreme.

A collection was also made in 2008 at the Greek village of Perissa on the island of Santorini (36° 21'10.04" N 25° 28'20.95" E). Fungal stromata were observed on a *B. tectorum* seed collected at this location.

The fungal structures of these Old-world isolates are visually similar to those found in the western US. They are recognizable on grass seeds due to the prominent

black stromata protruding out of the seed. When the observed organism is cultured on V8 juice agar medium it produces white mycelia that radiate out from the center of the plate. Following wounding, by scraping the mycelium with a sterile glass rod, the mycelium will produce stromata in a radial pattern on the V8 juice agar (Fig. 2). When cultured on modified alphacel medium (MAM) and wounded by scraping, the fungus sporulates and produces conidia on the mycelium. This behavior is consistent with that of *P. semeniperda* as described by Campbell et al. (2003).

A total of eight isolates from Love Valley were obtained from infected seeds. An isolate is a fungal culture that has been grown from a single spore taken from fungal stromata. More than one isolate can be obtained from the same seed due to multiple stromata growing from a seed, and preliminary data that we have collected shows that these separate stromata on the same seed can be due to infection from different strains. These isolates were sent to the Brigham Young University genetics lab and processed for genetic identification. Internal Transcribed Spacer, ITS, genetic sequencing analysis was used to identify the isolated fungus as *P. semeniperda*. The ITS sequence is about 500 base pairs; we have observed 13 different ITS genotypes for *P. semeniperda*. Some of the 13 ITS sequences vary from each other by as little as a single base. Of the eight Love Valley isolates, four of them align with the most common ITS sequence, Haplotype A (HTA). The HTA ITS sequence accounts for 50.4% of 437 genotyped isolates we have worked with from US populations. The other four isolates produced the ITS sequence, HTJ. The HTJ sequence was found in 2.1% of the isolates we have sequenced. This makes it uncommon but not unique since our data show that the sequence has been found at least at one western US study site.

The Greece isolate aligns with the HTC ITS sequence. We have only seen the HTC ITS sequence in one isolate from our Western US collections. This gives the HTC sequence a frequency of 0.84%, making it the rarest ITS sequence we have found. Due to the sequences differing by only a single base, HTA and HTJ and HTC match *P. semeniperda* 99% with the ITS sequence found on GenBank, a public database of nucleotide sequences maintained by the US National Center for Biology Information.

The Pamukkale, Turkey isolate was contaminated when the ITS sequencing was done, and we were unable to gain any uncontaminated genetic material. However, due to the morphological characteristics of this isolate on V8 agar we believe that it is also *P. semeniperda*.

The morphological similarities combined with the ITS sequence data provide strong evidence that we have discovered *P. semeniperda* in both Europe and Asia. The regions where collections were made fit with the CLIMEX model presented by Yonow et al. (2004). The CLIMEX model is produced with a software package by CSIRO Publishing, Melbourne, Australia, and is a popular method for assessing the risk of weeds, pests, and diseases. Their model predicts that the locations in which these collections were made are highly suitable for *P. semeniperda* to exist. It is reasonable to believe that with further searching the known range of *P. semeniperda* will be expanded.

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As Accepted for Publication

First Report of *Pyrenophora semeniperda* in the Old World

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Pyrenophora semeniperda (Brittlebank and Adam) Shoemaker (anamorph *Drechslera campanulata* (Lev.) Sutton) is a generalist seed pathogen that can cause high mortality in the seed banks of annual and perennial grasses. The current reported distribution of this pathogen is mainly temperate grasslands, deserts, and winter cereal-growing regions. It has been reported in Argentina, Australia, Canada, Egypt, New Zealand, South Africa, and the United States (3). *P. semeniperda* was originally described in France in the mid 1800's (1), but has not since been reported in Europe, and there are no known reports from Asia (4). Medd and Jones (3) expressed strong doubt that this fungus occurs naturally in the Old World. In May 2008, we observed what appeared to be *Pyrenophora semeniperda* on seeds from seed bank samples collected in Asia. Evidence of disease is readily observed as the development of macroscopic black fungal stromata protruding from the seed. The characteristic stromata were collected from a *Taeniatherum caput-medusae* seed near Pamukkale, Turkey and from six *Bromus tectorum* seeds in Love Valley, near Goreme, Turkey. An additional collection from a single *B. tectorum* seed was obtained from the Greek village of Perissa. Identity of the pathogen was tentatively confirmed by evaluating morphological characteristics of eight isolates from Love Valley, Turkey and one isolate from Perissa, Greece in V8 agar culture. After several days of incubation at 20°C with a 12-h light/dark regimen the cultures produced white mycelium that radiated out from the center of the plate.

Following wounding with a sterile glass rod, the cultures produced stromata in a radial pattern and conidiophores bearing distinctive large, crescent-shaped multi-celled conidia. These attributes are consistent with those of *P. semeniperda* as described by Campbell et al. (2). The identity of the nine Old World isolates as *P. semeniperda* was further confirmed using ribosomal DNA Internal Transcribed Spacer (ITS) genetic sequencing analysis. All nine isolates showed a 99% match with the *P. semeniperda* ITS sequence found on GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html), a public database of nucleotide sequences maintained by the US National Center for Biology Information. Pathogenicity of the Old World *P. semeniperda* isolates was confirmed by producing conidia in culture, dusting non-dormant *B. tectorum* seeds with 0.003g of conidial inoculum per 50 seeds, and incubating for 14 days at an alternating 10/20°C in a 12-h dark/light regimen. Stromata developed on >90% of inoculated seeds, and mortality as high as 34% was observed. Morphological similarities combined with ITS sequence data provide conclusive evidence that we have discovered *P. semeniperda* in both Europe and Asia. It is reasonable to believe that with further searching the known range of *P. semeniperda* will continue to be expanded.

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Figures for Discovery of *Pyrenophora semeniperda* in the Old World



Figure 1: Six *B. tectorum* seeds that are infected with *P. semeniperda*



Figure 2: *P. semeniperda* in V8 culture. Notice the radiating growth form and fungal stromata.

Virulence Variation in the Seed Bank Pathogen *Pyrenophora semeniperda*

(Prepared for submission to *Phytopathology*)

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Abstract

The generalist pathogen *Pyrenophora semeniperda* is an important pathogen of grass seeds in semiarid regions. It is particularly abundant in seed banks of the weedy annual grass *Bromus tectorum*. The pathogen is most active in spring, when *B. tectorum* seeds are in a state of secondary dormancy. It appears from field studies to have limited ability to kill nondormant seeds in the autumn seed bank. In this investigation, we measured virulence, defined as the ability to kill nondormant *B. tectorum* seeds, for 92 *P. semeniperda* isolates from 19 populations. Pathogen-caused mortality on nondormant seeds ranged from 0 to 44% and averaged 17.6%. High virulence (>30% mortality) was rare, occurring in only two isolates. Most of the variation in virulence was distributed among isolates within populations ($P < 0.0001$), with no significant among-population effect. There was no significant relationship between molecular marker (ITS) genotype and virulence phenotype, suggesting that virulence may evolve relatively rapidly in this organism. Virulence was significantly negatively correlated with mycelial growth rate, indicating that there may be a resource tradeoff between growth and production of toxic metabolites that confer virulence. Highly virulent forms may be at an adaptive disadvantage in competition with faster-growing isolates that can utilize seed reserves more quickly. Such highly virulent isolates may be useful in development of

mycoherbicides for quick knock-down of annual grass weed seed banks, especially as these isolates may not be able to persist long term in genetically diverse pathogen populations.

Keywords: *Bromus tectorum*, *Bromus rubens*, cheatgrass, downy brome, *Drechslera campanulata*, mycoherbicide, red brome, seed pathogen

Introduction

The use of fungal pathogens for the biocontrol of weeds has a long and complex history (TeBeest 1991), but methods using pathogens that target the seed stage are poorly developed (Chee-Sanford et al 2006). This is true even though it is currently thought that control at the seed stage, particularly of weedy annual grasses, is critical in management (Gallandt 2006). Ideally, successfully targeting the seed bank of an annual weed has the effect of drastically lowering plant densities. The seed bank dynamics of a plant can be greatly influenced by microorganisms in nature and, it offer potential to be developed for biocontrol (Kremer 1993, Chee-Sanford et al. 2006). In the present work, we examine the importance of variation in pathogen virulence in development of the seed pathogen *Pyrenophora semeniperda* (Brittlebank and Adam) Shoemaker (anamorph *Drechslera campanulata* (Lev.) Sutton) as a potential biocontrol organism for seeds of *Bromus tectorum* L. (cheatgrass, downy brome).

Bromus tectorum is an invasive winter annual grass from Eurasia that is largely responsible for an increase in frequency and size of fires on western North American semiarid wildlands. These fires destroy native plant communities, reduce biodiversity, and have major economic impacts, both direct and indirect. Because of widespread near-monocultures of the highly flammable *B. tectorum*, major fires may occur every three to

five years instead of at historic intervals of 60-100 years (Whisenant 1990). The native plant communities affected by this change in fire frequency are often unable to recover and in many areas are lost. Over 41 million acres in North America have been invaded with significant loss of native vegetation (Whisenant 1990). This problem has stimulated interest in developing biocontrol agents that could be effective against *B. tectorum* in wildland settings (Kennedy et al. 1991, 2001, Meyer et al. 2001, Boguena et al. 2007).

Pyrenophora semeniperda is a seed pathogen that is often found in abundance in *B. tectorum* seed banks (Meyer et al. 2007). It is a generalist that is believed to be an obligate biotroph in nature, carrying out its entire life cycle on living seeds (Medd et al 2003). This pathogen has a very wide host range on grasses and has also been found to infect several dicot species (Medd 1992). It is rarely seen in the perfect state, but is commonly encountered as the anamorph during seed testing of cereal grains and other grasses (Yonow et al 2004). It is readily recognized as macroscopic, fingerlike black stromata that protrude from infected seeds.

In recent years *P. semeniperda* has received considerable attention as a possible mycoherbicide for annual grass weeds that are a major problem in winter cereal crops (Medd et al 2003). It has been considered a weak pathogen because it often fails to kill infected seeds, which may continue to develop into normal seedlings (Campbell and Medd 2003). The majority of earlier work with this fungus focused on fast-germinating crop seeds. Furthermore, efforts to induce infection involved inoculating immature seeds in the inflorescence. Medd and Campbell (2005) concluded that this ‘floral’ infection was the primary infection mode leading to seed mortality. They carried out a field comparison of inoculum types and found that an inoculum of conidial suspension resulted

in greater floral infection than an inoculum of mycelium fragments. For *Bromus diandrus* L., Roth, seed mortality as high as 70% was reported.

In contrast to the results reported by these earlier workers, our studies with *P. semeniperda* have demonstrated its ability to infect and kill mature *B. tectorum* seeds after dispersal (Beckstead et al. 2007). We found that *P. semeniperda* is better able to kill slowly-germinating seeds, while rapidly germinating seeds usually escape mortality. We hypothesized that this differential mortality was due to a “race” for the stored endosperm reserves. If *P. semeniperda* is able to catabolize the starchy endosperm before the germinating seed can utilize it, then the seedling is greatly compromised in its ability to germinate. However, if the seed germinates quickly enough, *P. semeniperda* does not have time to consume the resources and the seed escapes death. This suggests that the primary target of the pathogen is slow-germinating secondarily dormant seeds in the spring seed bank, a hypothesis supported by field seed bank studies (Meyer et al. 2007). It also implies that rapid pathogen germination and growth should increase mortality on nondormant *B. tectorum* seeds.

The ability of some isolates of *P. semeniperda* to kill rapidly germinating, nondormant *B. tectorum* seeds could be an important aspect of development of this organism for biocontrol. Isolates of *P. semeniperda* are known to vary in virulence, and the degree of virulence has been related to levels of production of toxic metabolites in liquid culture. Virulence in these experiments was measured using leaf spot and wheat seedling bioassays, rather than the ability to kill nondormant seeds (Campbell et al. 2003). Biochemical studies have shown that some isolates of *P. semeniperda* produce large quantities of the cellular toxin cytochalasin B in culture, as well as more unusual

forms of cytochalasin (Capió et al. 2004, Evidente et al 2002). Our goal in the present study was to evaluate levels of virulence among diverse strains of the pathogen from a range of environments, using the ability to kill rapidly germinating, nondormant *B. tectorum* seeds as an index of virulence.

Our study objectives were: (i) to measure variation in the ability of *P. semeniperda* to kill non-dormant *B. tectorum* seeds, (ii) to examine the distribution of this virulence variation with regard to population and habitat of origin as well as genetic identity as determined by ribosomal DNA internal transcribed spacer (ITS) genotype, and (iii) to determine the relationships between variation in virulence and variation in mycelial growth rate, conidial germination, and conidial yield in culture. We hypothesized that there would be higher virulence in isolates from populations in mesic environments, where most *B. tectorum* seeds germinate rapidly in fall and very few carry over to form a dormant spring seed bank (Meyer et al. 2007). Based on the fact that the sexual stage of this organism is rarely observed, we expected molecular genotype and virulence phenotype to be strongly associated, because most traits should be linked in an organism that is largely clonal. Lastly, based on our earlier demonstration that the infection process in this pathosystem is essentially a race that can be won by rapidly germinating seeds (Beckstead et al. 2007), we hypothesized that factors associated with speed and high vigor in the pathogen (fast mycelial growth rate, high conidial yield, and high conidial germination) would be positively associated with the ability to kill nondormant seeds.

Materials and Methods

Isolation and Conidial Production. *Pyrenophora semeniperda* isolates were obtained from 19 populations during the course of two years of experimental work. These populations were collected from sites across Utah, Nevada, Idaho, Colorado, and Arizona (Table 1). We also obtained isolates from a population in Turkey, outside the previously reported range of this organism (Stewart et al. in press). Isolations were made from field-collected *P. semeniperda* stromata found to be infecting *B. tectorum* seeds in the seed bank (*Bromus rubens* L. seeds for two populations). Isolations were made using tweezers to pluck stromata from a seed. Stromata were then surface-sterilized by submersion for 60 sec in 70% ethyl alcohol (ETOH), 60 sec in 10% bleach, 60 sec in 70% ETOH, followed by rinsing with sterile deionized water (DI H₂O) for 30 sec to remove the ETOH and bleach. Stromata were then placed onto V8 agar medium in plastic Petri dishes (60x15mm) (Beckstead et al. 2007). They were stacked in translucent plastic bags and allowed to grow at 21°C (room temperature) under ambient cool-white fluorescent lighting with a 12-hour photoperiod. After one week of growth on V-8 agar, *P. semeniperda* colonies were wounded to promote more vigorous growth (Campbell et al. 2003b) using an L-shaped glass rod. This wounding was accomplished by adding 1ml of sterile Tween 80 (ICI Americas, Inc.) solution as a surfactant to each plate and scraping off the mycelial growth with the glass rod. The Tween 80 solution was prepared by adding 5 ml of sterile 1% Tween 80 to 200 ml of sterile DI-H₂O. The dishes were then placed back into their bags and the cultures were allowed to grow for two more weeks or until the stromata were long enough to isolate, about 0.5cm.

Freshly-cultured stromata were then transferred to dishes containing Modified Alphacel Medium (MAM). This was done by plucking a single stroma from a V-8 plate

with a pair of tweezers and placing it onto a MAM plate. From 5 to 20 MAM plates were made for each isolate, depending on the particular experiment in progress. The plates were placed in a bag and grown for one week. Again, the scraping procedure was carried out to increase conidial production. The plates were grown under a combination of 40w cool white fluorescent lights and black lights, with a 12 hour light-dark cycle, for 3-5 days (Campbell et al. 2003b). Conidial harvest took place by spraying the conidia from the Petri dishes into a 500ml beaker using sterile DI-H₂O. The conidial suspension was then filtered through a 25mic sieve. Conidia trapped on the sieve were allowed to dry for 24 hours. They were then scraped from the sieve using a rubber policeman, weighed to obtain conidial yield data, and placed into a sealed vial for storage at room temperature until the initiation of the virulence trials.

Conidial Viability Evaluation. Conidial viability was assessed for each isolate in a 24-hour germination test at room temperature (21C). To accomplish this, a sterile dissecting needle was dipped into a vial containing conidia and the attached conidia were suspended in 1ml of sterile DI-H₂O. This mixture was agitated and then spread over a water agar microscope slide. Conidia were incubated for 24 hours after which conidia were marked as germinated or non-germinated. Two replicates of 100 conidia for a total of 200 conidia were counted. The ratio of germinated to non-germinated conidia was then converted to a viability percentage. Results of the conidial germination test were analyzed using simple and multiple regression, with collection age and conidial yield per plate as independent variables and conidial germination percentage as the dependent variable.

Initial Virulence Screening. The virulence of all available *P. semeniperda* isolates (n = 92, Table 1) was evaluated by challenging nondormant *B. tectorum* seeds gathered at White Rocks in Skull Valley, Utah. Conidial inoculum (0.003g) was measured out for each of two replications of 50 seeds for each isolate. Seeds and conidia were placed in test tubes, which were then closed with rubber stoppers and agitated for 60 seconds using a modified sander to disperse the inoculum onto the seeds. Inoculated seeds were then placed in Petri dishes on the surface of two wet germination blotters (Anchor Paper, St. Paul Minnesota) to incubate under conditions reported optimal for mycelial growth (25C with an alternating photoperiod of 12 hours using white and black lights, as outlined in Campbell et al. 2003b). We included an uninoculated control, with seeds vibrated and incubated for germination but with no inoculum applied. Under these conditions, *B. tectorum* seeds are capable of germinating to >95% in less than 2 days (Allen and Meyer 2002). Following incubation for fourteen days to allow time for pathogen stomatal development, seeds were scored as germinated (radicle emergence to >1 mm) or killed (visible pathogen stomata present). No viable ungerminated seeds were observed at the end of the test. Results of the initial virulence screening were analyzed using simple and multiple regression with conidial germination percentage, collection age, and conidial yield per plate as independent variables and seed mortality percentage as the dependent variable.

Repeated Virulence Experiment. We used information on conidial viability and yield per plate to select a subset of 49 isolates from the original screening to be tested again (table 1). The isolates were chosen on two criteria: (i) >60% conidial germination, and (ii) relatively high yield per plate, resulting in sufficient conidia to repeat the

experiment. The conidial viability percentage for each isolate was used to correct the inoculum amounts so that 0.003g of live conidia were inoculated onto each replication of 50 non-dormant *B. tectorum* seeds. This subset of 49 isolates was then tested for virulence using the protocols from the initial screening.

Data for the 49 isolates included in both repetitions of the virulence screening were combined for statistical analysis. We first repeated the simple and multiple regression analyses performed on the original screening data set. We then performed three different nested mixed model analyses of variance (ANOVAs), each with a different fixed variable. Repetition in time was included as a random variable and isolate as a random variable nested within the fixed variable in each analysis. The fixed variables were population, habitat (mesic versus xeric) and ITS genotype (Boose et al, in review). We also classified the 49 isolates according to their mean virulence (% mortality of nondormant *B. tectorum* seeds) into nine virulence categories, and plotted the frequency of isolates in each of these categories in order to obtain a virulence frequency distribution.

Mycelial Growth Rate Studies. Mycelial growth rate experiments were carried out as described in Campbell et al. (2003), with a few modifications. First, we selected four isolates for a pilot experiment. We marked the undersides of 92mm x 16mm Petri dishes with equidistant transects at 45° intervals. The dishes were filled with 15-20ml of 1/4 strength potato dextrose agar. We then transferred a single germinated conidium to the center of each Petri dish and allowed the mycelium to grow at room temperature (ca. 21C) for 14 days. Radius lengths for each of ten replicates of the four isolates were measured at 3, 5, 7, 11, and 14 days, with eight measurements per plate per

read day. We examined these pilot data using analysis of covariance with isolate as the categorical variable, days of incubation as the continuous variable, and colony radius as the response variable, and we also regressed day 14 colony diameter (2x radius) on the slope of increase in colony diameter through time (growth rate)

From this preliminary experiment, we determined from this analysis that we could achieve sufficient precision with five replications per isolate and with day 14 colony diameter as a surrogate for growth rate. We then chose 18 isolates representing a range of virulence and repeated the experiment with the above simplifications. To establish the relationship between growth rate and virulence, we regressed virulence on mean day 14 colony diameter.

Results

Conidial Viability Evaluation. Mean conidial germination was 73.9% with a range of 2-98%. Germination percentage was significantly negatively correlated with post-collection age of the conidia, which explained 25% of the variation in conidial germination (fig. 1A). This effect was probably due to viability loss in storage, as post-collection age ranged from a few weeks to two years. Germination percentage was also negatively correlated with conidial yield, but this independent variable explained only 7.5% of the variation (fig. 1B). Some low-yielding isolates have been observed to produce malformed conidia, which may be less competent to infect seeds. Both post-collection age and yield per plate were significant in multiple regression, explaining 35.2% of the variation in conidial germination percentage (table 2).

Initial Virulence Screening. Mean seed mortality (virulence) in the initial screening with all available isolates (n = 90) was 12.2%, with a range of 0-44%.

Virulence was found to be significantly positively correlated with conidial germination percentage (fig. 2A) and conidial yield per plate (fig. 2B), and negatively correlated with post-collection age (fig. 2C). All three variables were significant in multiple regression, suggesting that the effects of post-collection age and yield per plate on virulence were at least partly independent of their effects on conidial germination (table. 3). The three variables collectively accounted for only 26.8% of the variation in virulence, indicating that virulence variation could not be solely due to the effects of proximal environmental factors during conidial production and storage.

Repeated Virulence Experiment. Mean seed mortality (virulence) averaged across both repetitions in time for 49 isolates was 17.5% with a range of 1.5-43.3%, values similar to those from the initial screening. Virulence in the combined data set was no longer significantly correlated with either conidial germination or yield per plate. Thus the impact of these variables on virulence expression was effectively removed by eliminating isolates with low germination and low conidial yield. Virulence was still significantly correlated with post-collection age (d.f. = 47, $R^2 = 0.233$, $P < 0.0001$). Older conidial collections included in the repeated trials were often observed to germinate more slowly, as evidenced by their shorter germ tubes after 24 hours, even though they were able to achieve the threshold germination percentage of 60%.

Most of the variation in virulence was distributed among isolates within populations ($F = 8.77$, d.f. = 32,146, $P < 0.0001$). The differences among populations were not significant ($F = 0.69$, d.f. = 16, 32, $P = 0.7849$). Similar results were obtained when ITS genotype or habitat (mesic versus xeric) was included in the model as a main effect in place of population. There was no significant difference in virulence among ITS

genotypes or between mesic and xeric habitats, largely because of the large and significant amount of variation among isolates within each of these main effect categories.

The frequency distribution for virulence was strongly right-skewed, with many low-virulence isolates and very few high-virulence ones (fig. 3). The modal virulence category was 21-25%, while both the median and mean values fell in the 16-21% category. Over 57% of the isolates fell into the four categories below the mode, with only about 16% in the four categories above the mode. This indicates that high virulence is relatively rare, at least in the set of isolates included in this study. Most isolates exhibited low to intermediate virulence.

Mycelial Growth Rate Studies. In ANCOVA for mycelial growth rate of four isolates we found that colony diameter increased as a linear function of time ($F = 2337$, d.f. = 1, 19, $P < 0.0001$). The slopes (growth rate in mm/day) were significantly different among isolates ($F = 27.63$, d.f. = 3, 19, $P < 0.0001$). Growth rate and day 14 colony diameter were almost perfectly correlated ($R^2 = 0.992$). Thus, we were able to use day 14 colony diameter as a surrogate for growth rate.

Among 18 isolates representing a range of virulence, day 14 colony diameter varied from 42 to 72mm. Contrary to our hypothesis, the relationship between growth rate (day 14 colony diameter) and virulence was negative (fig. 4). The slowest growing isolates were the most virulent, and the fastest growing isolates were the least virulent.

Discussion

We found considerable variation in the ability of *P. semeniperda* to kill non-dormant *B. tectorum* seeds (mortality range 0-44%). This variation was most significant

within populations, not among populations, possibly because inadequate sample size for most populations restricted our ability to detect among-population differences (Table 1). High virulence (>30% kill) was rare and limited to a single population, Tenmile Creek, Utah, where two highly virulent isolates were identified. The virulence frequency distribution was strongly right-skewed, with most isolates expressing relatively low levels of virulence.

Factors other than intrinsic virulence that affected ability to kill non-dormant seeds in the initial screening included post-collection age of conidial inoculum, conidial germinability, and ability to produce abundant conidia in culture. As conidia aged, they gradually lost their ability to germinate. Storage under suboptimal conditions, such as at higher than optimal moisture content, may also have been a factor in causing some conidial collections to lose viability. Ten of the older conidial collections in this experiment were also tested for virulence in an early pilot experiment when recently harvested. Over two years, these isolates lost an average of 91% of their ability to kill non-dormant seeds. Five of them were no longer able to kill non-dormant seeds, and only one was still as virulent in this trial as it was in 2007. Mean expressed virulence for these ten isolates dropped from 11% to 1% during two years of laboratory storage.

Because both the pathogen and the seed compete for endosperm reserves (Beckstead et al 2007), if the conidia lose their ability to germinate quickly they will not be successful in using the resources before the seed uses them. Nondormant *B. tectorum* seeds germinate very quickly. The importance of rapid conidial germination is further demonstrated by the fact that, even after inoculum load was increased to adjust for viability in the second repetition, a significant increase in seed mortality was not

observed. Increasing the density of live conidia on a seed will increase the number germinating, but it cannot make the conidia germinate faster.

To obtain isolates that are capable of expressing their maximum level of intrinsic virulence, optimal cultural conditions are needed. These conditions apparently vary by isolate. Ability to produce conidia in culture, measured as conidial yield per plate, varied among isolates and was significant in explaining variation in virulence. Many of the low yielding isolates also produced malformed and probably less fit conidia, which may have been responsible for their poor performance in the virulence trials. It is possible that these isolates may perform quite differently under field conditions.

Conidial germination percentage, conidial yield per plate, and conidial collection age are inter-related variables that deal with proximal environmental factors. These factors modify the way in which virulence is expressed in different isolates by mediating the ability of an isolate to germinate and infect quickly. These variables are probably artifacts of our conidial production methods and storage conditions. Under natural conditions, all of our tested isolates are likely capable of producing normal quantities of highly viable conidia. In fact, we had many isolates that did not sporulate at all under our cultural conditions; these isolates were obviously not included in the virulence trials.

The strongly right-skewed frequency distribution for virulence (fig 4) suggests that high virulence either has little adaptive advantage or a high fitness cost, or both. The negative relationship between virulence and mycelial growth rate indicates a fitness tradeoff. This negative correlation suggests that if the isolate grows faster it is putting fewer resources into producing phytotoxic metabolites that are positively correlated with virulence (Campbell et al 2003a). These metabolites are known to retard seedling

growth, and it is reasonable to hypothesize that an isolate that can produce high levels of these toxic metabolites can prevent a seed from germinating quickly. Once germination is slowed, the pathogen can deplete the endosperm resources, killing the seed. To test this hypothesis, we would need to determine if slower growing isolates produce higher levels of these metabolites when compared to faster growing isolates.

We hypothesized that there would be a selective advantage for a highly virulent strain in a mesic environment, where all seeds are nondormant in the fall and most germinate in the first germination event leaving few to none in a secondarily dormant (slow germinating) state. This was not confirmed. The pathogen can sporulate on germinated seeds, so that killing the seed is not necessary in order to reproduce, but killing the seed should improve fitness by increasing reproductive output (i.e., number of conidia produced). This is not what we observed. We know that multiple pathogen ITS genotypes can infect and sporulate on a single seed (Clement unpublished data). The tradeoff between virulence and growth rate would mean that the strain that prevents the germination of a nondormant seed is not necessarily the one exploiting the resources, because fast growing, co-infecting strains may cash in more quickly and usurp the seed resources made available by the more virulent strain. Also, on dormant seeds high virulence presents no fitness advantage, because any strain can kill slow-germinating seeds before they germinate. In multiple-infection scenarios on dormant seeds the high-virulence isolate should be at a selective disadvantage, because its slow growth rate gives more time for a faster growing co-infecting strain to use the available resources. This helps explain why high virulence is rare, but leaves unanswered the question as to why highly virulent isolates can be found at all.

There was no significant difference in virulence among the ITS genotypes. ITS markers are a coarse genetic marker, and many genetically different isolates can fall within a single ITS genotype. If a marker system with finer resolution were used, for example, SSR (single sequence repeat) markers, a difference in virulence among different genotypes might be detected. The fact that high virulence seems to occur at random with respect to population, habitat, and ITS genotype tends to suggest that it represents a maladaptive product of mutation or more likely recombination that would persist for a limited period of time and eventually be selected against in competition with less virulent, faster-growing strains. The mechanism for variation in virulence needs to be established; we hypothesize that it is related to the levels of production of one or more toxic metabolites, probably cytochalasins. This brings about the need to explore the genetic regulation of the biosynthetic pathway that produces cytochalasin in this organism. This exploration could bring us closer to understanding how sexual recombination can result in the appearance of a potentially maladapted highly virulent strain. It could also lead to the ability to breed for hypervirulent strains for use in biocontrol.

While the genetic characterization of virulence is being pursued, we also need to continue with a more empirical approach. There is no reason to assume we have discovered the most virulent strain of *P. semeniperda*, and strains with higher virulence may yet be encountered. High virulence could be a useful trait in a mycoherbicide that quickly knocks out a high percentage of non-dormant seeds of an annual host before they can germinate. The fact that highly virulent strains are probably less fit than less virulent strains is a plus in that a highly virulent strain deployed as a mycoherbicide would

probably not be able to persist long term. Further, rapid-growing and virulent strains could be combined during inoculum production, in order to more effectively compromise the seedling's ability to successfully establish.

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Table 1. Location, host, and site information for nineteen *Pyrenophora semeniperda* populations included in virulence trials.

Population	State	Lat (N)	Long (W)	Elev. (m)	Mean Annual precip. (mm)	Number of Isolates	
						Initial screen	Repeated trials
On <i>B. tectorum</i> at mesic sites in North America							
Dinosaur	CO	40.387	-108.996	2372	404	6	3
Dry Fork Face	UT	40.598	-109.662	2609	408	4	2
Dutch John	UT	40.933	-109.416	1954	330	5	2
Jim Creek	NV	41.318	-115.901	1928	336	5	2
Milk Ranch	UT	37.666	-109.720	2302	519	4	1
Strawberry	UT	40.227	-111.124	2332	510	4	3
White's Valley	UT	41.808	-112.303	1508	439	3	2
West Mountain	UT	40.138	-111.804	1393	508	1	1
On <i>B. tectorum</i> at xeric sites in North America							
Bruneau	ID	42.843	-115.758	784	207	3	2
Cinder Cone Butte	ID	43.221	-115.993	1038	270	2	2
Dog Valley	UT	39.716	-111.956	1729	396	6	1
Gusher	UT	40.303	-109.773	1565	177	3	1
House Range	UT	39.231	-113.288	1773	281	4	0
Independence Valley	NV	41.041	-114.749	1741	290	2	1
Tenmile Creek	UT	41.865	-113.136	1459	251	10	8
Whiterocks	UT	40.328	-112.778	1449	203	11	5
On <i>B. rubens</i> at xeric sites in North America							
Mormon Mountains	NV	36.844	-114.364	953	310	2	0
Pakoon Valley	AZ	36.563	-113.942	1164	156	7	4
On <i>B. tectorum</i> at a xeric site in Turkey							
Middle Love Valley	----	38.659	34.821	945	205	10	9
Total Isolates						92	49

* Precipitation data: <http://www.prism.oregonstate.edu/>

Table 2. Multiple regression analysis for the relationship between conidial germination percentage and two independent variables, $R^2=0.3519$, $F=24.16$, $d.f.=2,89$, $p<0.0001$.

Parameter	DF	Parameter Estimate	SE	t-value	p-value
yield per plate (g)	1	1711.88	460.34	3.72	0.0003
collection age (days)	1	-0.0712	0.0115	-6.17	<0.0001
intercept	1	81.08	3.186	25.44	<0.0001

Table 3. Multiple regression analysis for the effects of three independent variables on the virulence of an isolate, $R^2=0.2678$, $F=10.73$, $d.f.=3,88$, $p<0.0001$.

Parameter	DF	Parameter Estimate	SE	t-value	p-value
conidial germination percentage	1	0.095	0.0461	2.06	0.0421
yield per plate (g)	1	464.01	215.09	2.16	0.0337
collection age (days)	1	-0.0159	0.006	-2.65	0.0096
intercept	1	6.542	3.984	1.64	0.1042

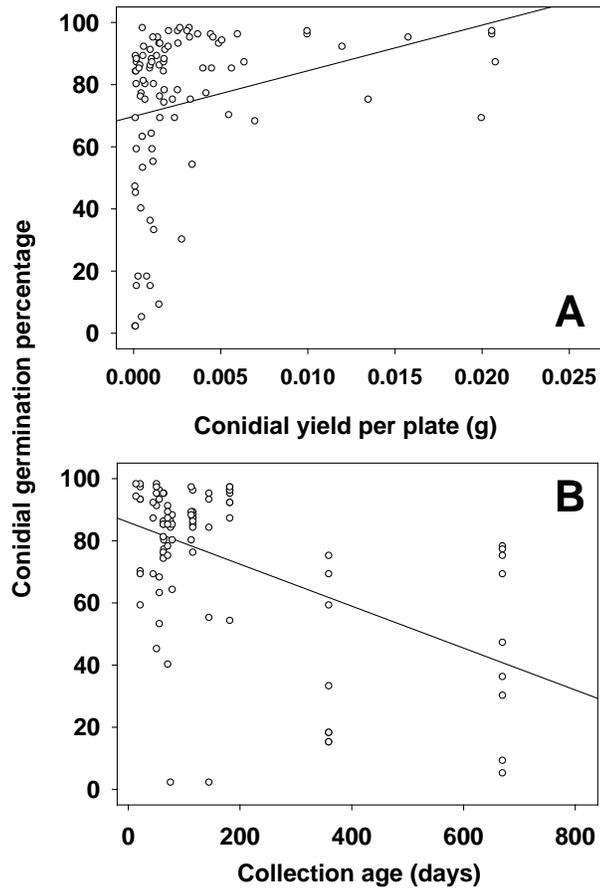


Fig. 1. The relationship of conidial germination percentage to (A) conidial yield per plate, $\text{germination} = 1469.17(\text{conidial yield per plate (g)}) + 69.77$, $R^2 = 0.0747$, d.f.=91, $p = 0.0084$, and (B) collection age, $\text{germination} = -0.068(\text{collection age (days)}) + 85.95$, $R^2 = 0.2512$, d.f.=91, $p < 0.0001$

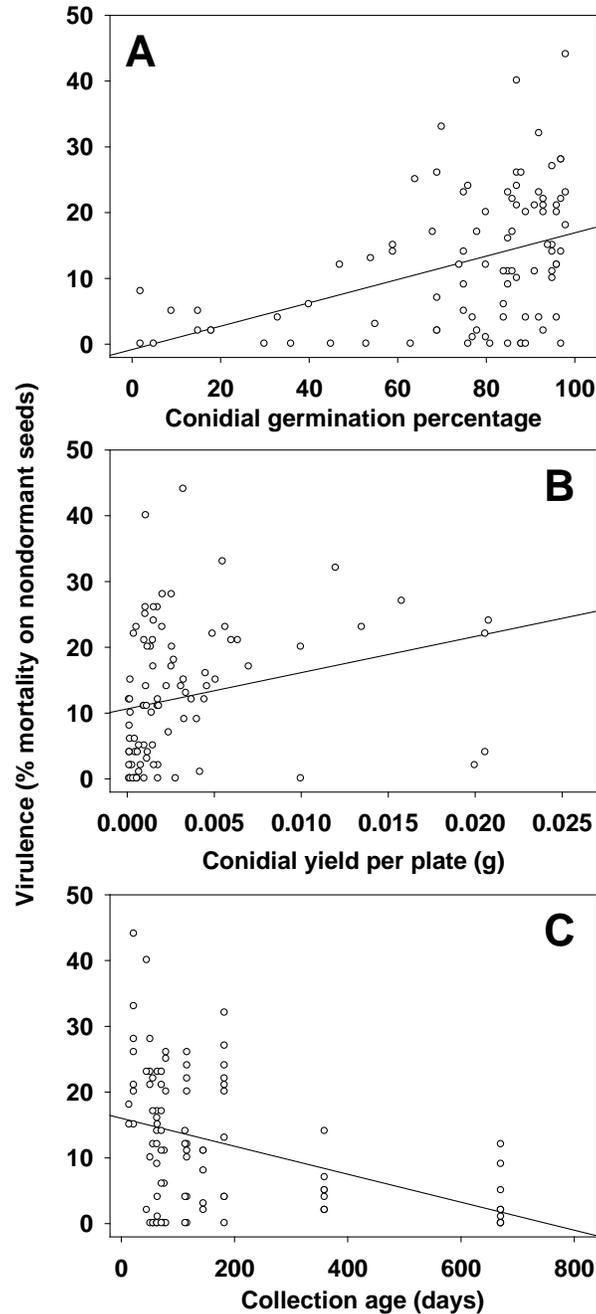


Fig. 2. Relationship of virulence to (A) conidial germination percentage, $\text{virulence} = 0.1776(\text{conidial germination percentage}) - 0.8153$, $R^2 = 0.1907$, d.f.=91, $p < 0.0001$, (B) conidial yield per plate, $\text{virulence} = 549.54(\text{conidial yield per plate (g)}) + 10.65$, $R^2 = 0.0632$, d.f.=91, $p = 0.0156$, and to (C) collection age, $\text{virulence} = -0.0213(\text{collection age (days)}) + 16.03$, $R^2 = 0.1509$, d.f.=91, $p = 0.0001$.

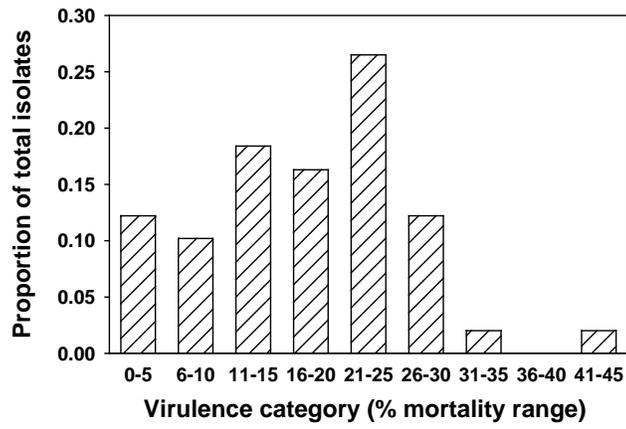


Fig. 3. Proportion of total isolates in each of nine virulence categories, n = 49 isolates.

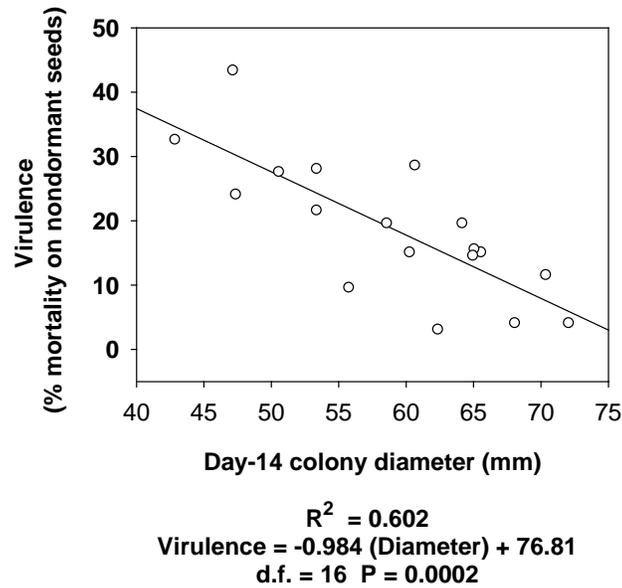


Fig. 4. Relationship of virulence to day 14 colony diameter (mm), n=18. Isolates used represent a range in virulence. Virulence=-0.984(diameter (mm))+76.81, $R^2=0.602$, d.f.=16,p=0.0002.

Appendix A

Isolation Method: Whole Stromata Isolation

The following steps provide a detailed isolation procedure for whole stromata isolation.

1. Using tweezers pluck stromata off from seed.
2. Sterilize stromata.
 - Submerge single stromata for 60 sec. in 70% ethyl alcohol. (ETOH), 60 sec. in 10% bleach, 60 sec in 70% ETOH, rinse with sterile deionized (DI) H₂O for 30 sec to remove the ETOH and bleach.
 - If sterilizing a large quantity of stromata place filter paper in a 125 micron mesh sieve and place stromata on top of paper. Then follow the above process dipping the sieve.
3. Place sterile stromata into Petri dish (60x15mm) containing V8 agar.
4. Place dishes into sealed bags.
5. Incubate for 7 days under inflorescent lighting (12 hour light dark periods).
6. Scrape dishes.
 - Add sterile 1% Tween 80 solution to sterile DI H₂O, 1ml Tween per 100ml H₂O.
 - Add above solution to V8 dishes, 5ml.
 - Sterilize a bent glass rod.
 - Using the rod scrape the mycelium off from dish into a large beaker.
 - Re-sterilize the rod after every 5 dishes.

- Place dishes back into bags.
7. Incubate for 14-20 days (or until stromata are big enough to pick off of the agar with tweezers) under room lighting (12 hour light dark period).
 8. Place stromata from V8 Petri dish onto MAM Petri dish (60x15mm).
 9. Place dishes into sealed bags.
 10. Incubate for 7 days under fluorescent lighting 12 hour light-dark periods.
 11. Scrape dishes
 - Add sterile 1% Tween 80 solution to sterile DI H₂O, 1ml Tween per 100ml H₂O.
 - Add above solution to MAM dishes, 1 pipette full.
 - Sterilize a bent glass rod.
 - Using the rod scrape the mycelium from dish into a large beaker.
 - Re-sterilize the rod after 5 dishes or for each new isolate.
 12. Incubate for 3-5 days under white and black lights for a 12 hour photoperiod with dishes sitting on a flat surface. Do not stack dishes.
 13. Harvest conidia.
 - Sterilize 1 liter beaker.
 - Sterilize 500 ml DI H₂O.
 - Fill spray bottle with DI H₂O.
 - Holding the dish over the beaker, spray each MAM dish so that the conidia run into the beaker. Do this for all dishes of the isolate.
 - Filter conidia solution through a 25 micron mesh sieve.
 - Place sieve to air dry.

- When conidia are dry use a rubber policeman to scrap conidia off from sieve.
- Place conidia into an appropriate sized sealed container for storage.

Appendix B

Isolation Method: Single Conidia

The following steps provide a detailed isolation procedure for single conidia isolation.

- Method:
 1. Using tweezers break a stroma off of a field collected seed.
 2. Set stroma on a wet blotter for 24 hrs to allow it to produce conidia at the break point.
 3. Use dissecting needle to collect conidia and rinse off the needle in a 20 ml vial of sterile H₂O/Tween.
 4. If the conidia are thick on the dish, drag the needle through the conidia, rinse in vial and repeat.
 5. If the conidia are only on fingers in the dish, rub the needle around the finger until some of the conidia adhere to the needle. Then rinse off and repeat as many times as needed to obtain many conidia.
 6. Shake or vortex the vial to mix the conidia well in the water.
 7. Pour mixture onto water agar dish and gently swirl to evenly spread conidia around dish.
 8. Let sit 1-2 minutes to allow conidia to settle on surface of agar (especially when few conidia are present).
 9. Pour off excess water, set dish at tilt, and blot excess water with Kimwipe. (Fold the Kimwipe in half then again in thirds, then half again and absorb with corner).

10. Put Petri dishes in bag or wrap with parafilm until the next day (as close to 12 hours later as possible because conidia germinate quickly and the mycelium can intertwine).
 11. With the dish under the dissecting microscope find a single conidium far enough away from others to know that it is just a single one.
 12. Dip hyphal tipping needle in ETOH and flame to sterilize (re-sterilize before each conidial transfer).
 13. Transfer the piece of agar with single conidium to MAM dish.
 14. Incubate for 7 days under fluorescent lighting (12 hour light-dark cycle).
- Scrape dishes.
 1. Add sterile 1% Tween 80 solution to sterile DI H₂O, 1 ml Tween per 100 ml H₂O.
 2. Add above solution to MAM dishes, 5 ml.
 3. Sterilize a bent glass rod.
 4. Using the rod scrape the mycelium off from dish into a large beaker.
 5. Re-sterilize the rod after every 5 dishes.
 6. Incubate for 3-5 days under white and black lights with dishes sitting on a flat surface. Do not stack dishes.
 - Harvest conidia
 1. Sterilize 1 liter beaker.
 2. Sterilize 500 ml DI H₂O.
 3. Fill spray bottle with DI H₂O.

4. Holding the dish over the beaker, spray each MAM dish so that the conidia run into the beaker. Do this for all dishes of the isolate.
5. Filter conidia solution through a 25 micron mesh sieve.
6. Place sieve to air dry.
7. When conidia are dry use a rubber policeman to scrap conidia off from sieve.
8. Place conidia into a sealed glass container for storage.

Appendix C

Isolation Methods Data

Experiment 1

The ability to control contaminating agents and grow pure cultures of *Pyrenophora semeniperda* is an essential step in producing inoculum. Overcoming this obstacle was a major milestone in culturing isolates. To find the optimum conditions for conidial growth that also restricts contaminant growth a comparative experiment was carried out.

Isolates from five populations were obtained, according to the procedure in Appendix A, for each of the treatment categories. The five populations were White Rocks, Dog Valley, 10 Mile Creek, House Range, and Pakoon (see table 1 in chapter “Virulence Variation in the Seed Bank Pathogen *Pyrenophora semeniperda*”). Treatments varied include differing labs, presence or absence of black light, warm (25C) or cool (10C), and surface sterilization of stromata onto MAM or no surface sterilization onto MAM.

The isolates were visually scored on the following criteria: percent contamination on V-8 agar, percent conidia on MAM, percent stromata on MAM, and “tar” production on MAM. Tar production is when no stromatal or conidial growth has occurred, but *P. semeniperda* has grown flat and black over the MAM. Percentage was visually estimated as the proportion of the dish that was covered by stromata, tar, conidia, or contamination.

Temperature was important in its effects on the different populations and in the amount of contamination. When initially isolating stromata from field collected seeds onto V8 plates, cool temperature resulted in significantly ($P < 0.0001$) lower

contamination than warm temperature. Contamination significantly ($P < 0.0001$) varied by population while the interaction of population and temperature was also significant ($P < 0.0001$) (fig. 1A). In other words, the level of contamination was affected by temperature more for some populations than for others. Once isolates were transferred to MAM plates, low temperature remained significant ($P < 0.0001$) in controlling contamination. Contamination no longer varied significantly over populations, but the interaction remained significant ($P < 0.0001$) (fig. 1B). When isolates were allowed to sporulate on MAM, high temperature resulted in significantly ($P < 0.0001$) higher conidial production (fig. 1C) while cold temperature results in significantly ($P < 0.0001$) higher stromatal production (fig. 1D). Tar was significantly ($P < 0.0001$) increased in high temperature and was significantly ($P = 0.0006$) higher in some populations than others. Temperature and population significantly interacted ($P < 0.0001$) (fig. 1E). This meant that higher temperatures had a greater effect on tar production for some populations than others.

Due to the large sample size ($n = 2000$), the main effects and many of their interactions were statistically significant. However they usually only varied by small margins and apparently were not biologically significant. The results that were most important to answering the objective of this experiment were as follows: cold reduces contamination but increases stromatal production on MAM, and none of the isolates would sporulate directly on the mycelium in the cold. Tar was exhibited more in certain populations and at higher temperature. The effort and time that it takes to sterilize from V8 plates to MAM plates is not warranted because it did not result in reduced contamination (fig. 2).

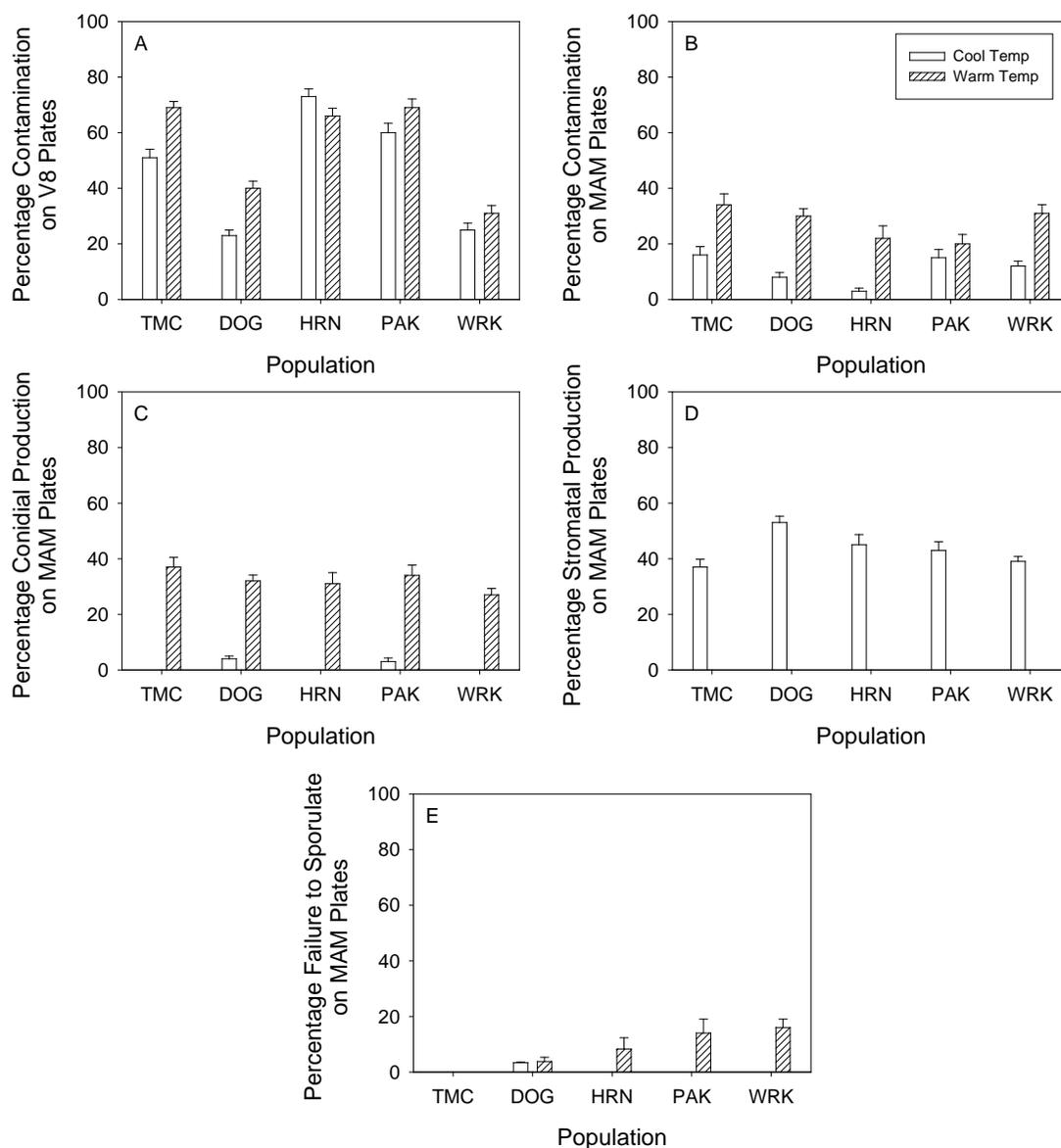


Fig. 1. (A) Percentage contamination on initial V8 isolation plates as a function of temperature and population (temperature $P < 0.0001$, population $P < 0.0001$, interaction $P < 0.0001$), (B) Percentage contamination after transfer to MAM (temperature $P < 0.0001$, population ns, interaction $P < 0.0001$), (C) Percentage of MAM plates with direct conidial production on mycelium (population ns, temperature $P < 0.0001$, interaction $P = 0.0005$), (D) Percentage of MAM plates with only stromatal production (temperature $P < 0.0001$, population ns, interaction $P < 0.0001$), (E) Percentage of plates with failure to produce either conidia directly on the mycelium or stromata (temperature $P < 0.0001$, population $P = 0.0006$, interaction $P < 0.0001$). Error bars above each bar indicate the standard deviation.



Fig. 2. Contamination percentage and the standard error on MAM plates following transfer of stromata produced in V8 agar culture as a function of whether or not the newly produced stromata were surface-sterilized prior to transfer to MAM (sterilization treatments did not significantly differ).

Experiment 2

This experiment was designed to test stromatal isolation compared with single spore isolation. Five populations were chosen and five isolates per population were made using each isolation method as outlined in appendices A and B. For each isolation method the number of clean dishes, contaminated dishes, and dishes that did not grow anything were counted. Conidial production (conidial yield in grams per culture plate) was also calculated and compared among isolates and isolation methods.

Single spore isolation resulted in more clean cultures from field stromata than stromata isolation did (fig. 1A). When isolating from stromata onto V8 plates, just over 40% of the plates were lost during the sterilization process, about 20% were lost due to contamination, and only about 38% remained clean and grew a culture. In contrast, the single spore method resulted in >95% clean cultures. This step is what makes single spore isolation quicker and more efficient than stromatal isolation. Once stromata from the V8 plates are transferred to MAM plates for conidial production there was no significant difference between the two methods (fig. 1B). The use of single spore isolation allows for faster production of cleaner cultures than the use of stromatal isolation.

When methods were compared at the population level, single spore isolation significantly ($P=0.0501$) produced cleaner cultures than stromata isolation (fig. 2A). Overall conidial yield per plate for each of the isolation methods were significantly ($P=0.0558$) different with single spore isolation, producing higher yielding plates than stromata isolation did (fig. 2B). However, when only the yield of harvested plates was counted, then the isolation method by population no longer was significant (fig. 2C).

Overall single spore isolation was cleaner, faster, and yielded more conidia than the stromata isolation method. Therefore, it is recommended to use the single spore isolation method for future isolation work.

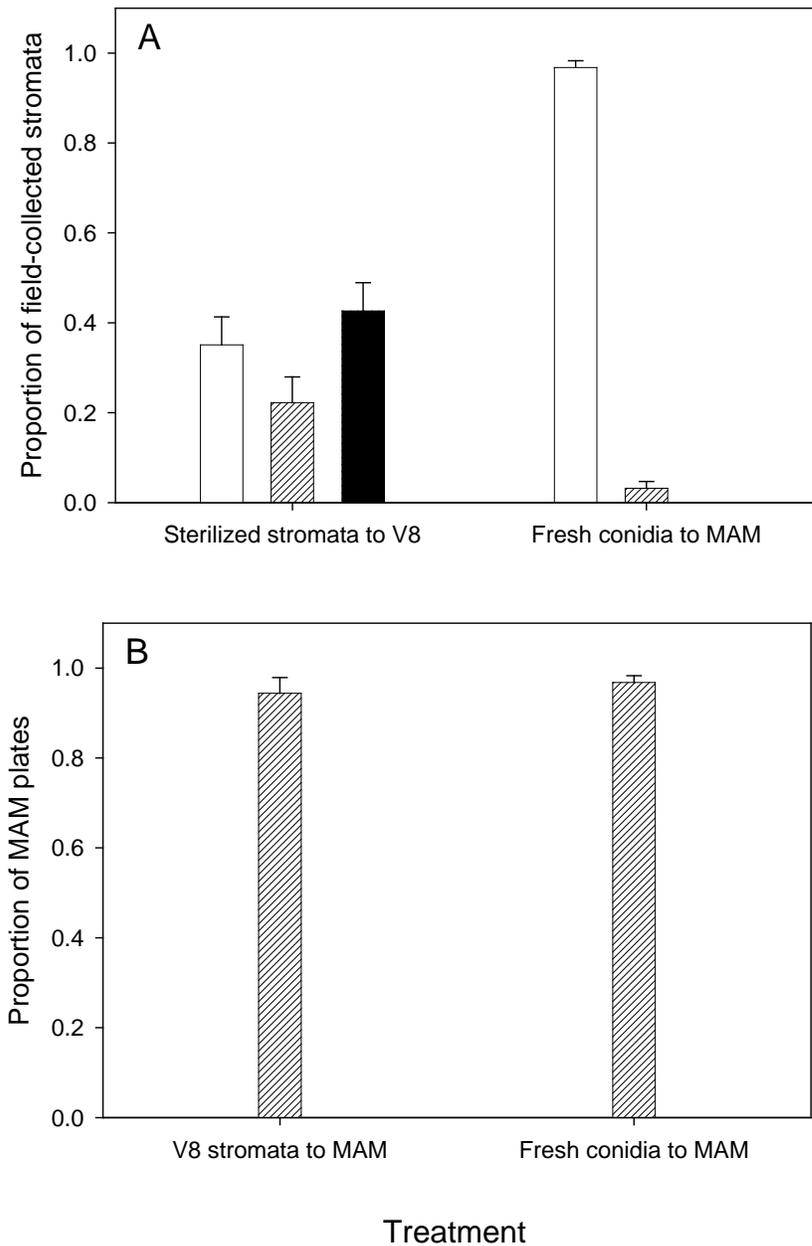


Fig. 1. (A) Proportion of stromata from field seed bank seeds that produced clean cultures (white), contaminated cultures (hatched), or failed to grow in culture (black) for two methods: surface-sterilization of the stromata followed by plating onto V8 agar and transfer of freshly produced conidia from a wounded finger directly to MAM ($P < 0.0001$). (B) Proportion of MAM plates that produced clean cultures from stromata incubated on V8 agar and transferred to MAM and from fresh conidia transferred directly to MAM (not significant).

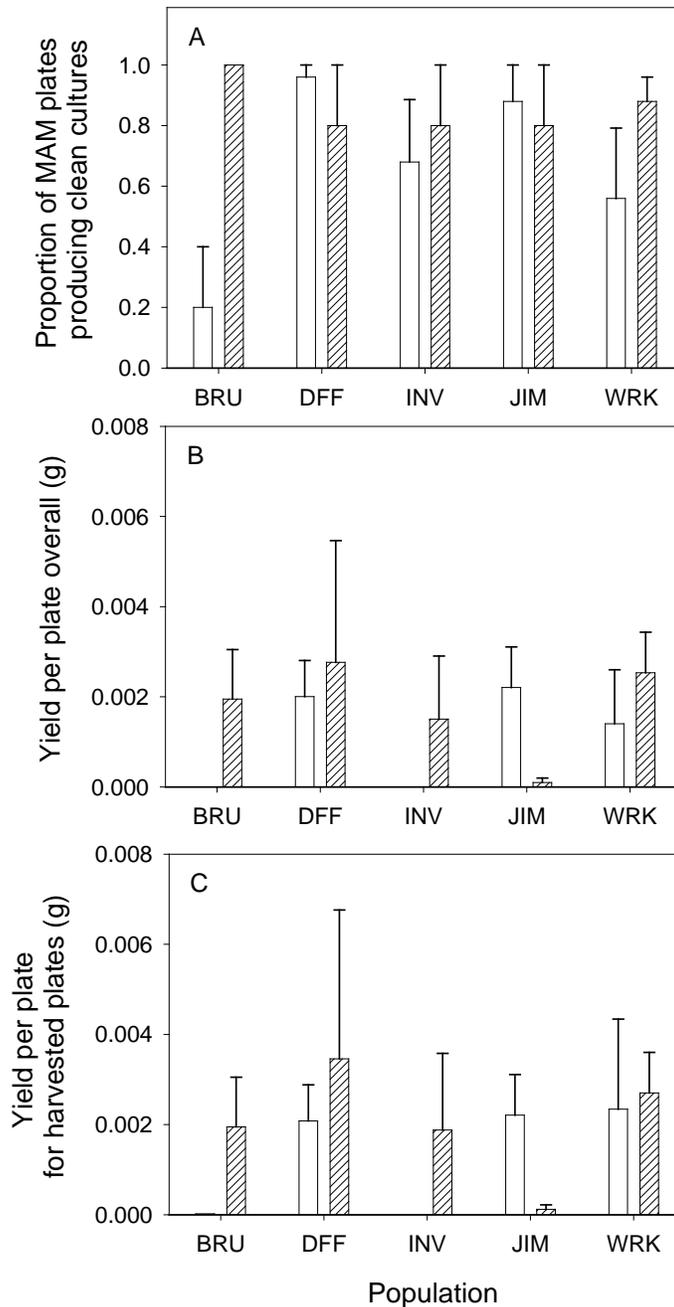


Fig. 2. (A) Proportion of MAM plates that produced clean cultures after wounding, for isolates from five populations produced by two methods as in Figure 1: sterilizing stromata (white) and isolating conidia directly from wounded stromata (hatched) (method main effect significant at $P=0.0657$, method by population interaction significant at $P=0.0501$). (B) Overall conidial yield per plate overall for five populations and two treatments (method by population interaction significant at $P=0.0558$). (C) Conidial yield per plate for harvested plates for five populations and two treatments (method by population interaction not significant).

Appendix D

Agar Recipe: Modified Alphacel Medium

To mix 5L take 50g of dry oatmeal, add 1L DI H₂O and autoclave the mixture at 100 °C for 80 min. Filter the oatmeal mixture and add the liquid to a 5L flask. Add 5g MgSO₄·7H₂O, 7.5g KH₂PO₄, 5g NaNO₃, 250ml coconut milk, 85g agar, DI H₂O to the 5L mark on the flask, heat to 65 C° while stirring continuously, this ensures proper mixing of ingredients. Autoclave and allow to cool before pouring into dishes.

Agar Recipe: V8 Juice Agar

To mix 2L combine 360ml regular V8 juice, 3.98g CaCO₃, 33g agar, DI H₂O to the 2L mark on the flask. Heat to 65 C° while stirring continuously. This ensures proper mixing of ingredients. Autoclave and allow to cool before pouring into dishes.

Appendix E

Field Trail Data

A field trial of suspended conidial inoculum was designed and carried out. The goal was to see if applying different amounts of conidial inoculum in the field would result in increased kill of *Bromus tectorum*. The experiment was installed at the White Rocks Exclosure in Skull Valley, Utah. A randomized block design was used with four different treatments and a control. Each block contained five square foot plots. The treatments were the application of fungicide, low inoculum (.02g dry conidia/150ml water), medium inoculum (.08g dry conidia/150ml water), and high inoculum (.16g dry conidia/150ml water).

These treatments were applied in the fall (September) before the first significant precipitation event. In the spring (May), before seed shatter, seed bank samples were taken from each plot. These seed bank samples were then processed by extracting all the non-germinated *B. tectorum* seeds from the samples. They were scored as viable seed or killed seed. A killed seed was one with fungal stromata from *Pyrenophora semeniperda*. The apparently viable seeds were then incubated and after 14 days scored again as viable or killed by the pathogen.

The results of this experiment were that 99.9% of carryover seeds were killed in all treatments. There was no treatment effect observed.

A second field trial was designed and carried out. It included two sites, one in Skull Valley, Utah and one in Santaquin Canyon, Utah. Each site was set up in a randomized block design with five square foot plots per block, and a total of ten blocks. The treatments were four different levels of inoculum and a control. The inoculum was a

solid carrier inoculum, mycelium grown in Agsorb (Agsorb Products Group) , dried, and crushed, with the four levels being, 5g, 15g, 30g, and 45g. This was installed in the fall (September) before any significant precipitation and seed bank samples were taken in the spring before seed shatter (May).

At the publication of this thesis the seed bank samples had not been processed for the second field trial. They will be processed in the same manner as the first field trial.