

Mycelial growth rate and toxin production in the seed pathogen *Pyrenophora semeniperda*: resource trade-offs and temporally varying selection

S. E. Meyer^{a*}, M. Masi^b, S. Clement^a, T. L. Davis^c and J. Beckstead^c

^aShrub Sciences Laboratory, US Forest Service Rocky Mountain Research Station, Provo, UT 84606; ^bDepartment of Plant and Wildlife Sciences, Brigham Young University, Provo, UT 84606; and ^cDepartment of Biology, Gonzaga University, Spokane, WA 99258, USA

Pyrenophora semeniperda, an important pathogen in *Bromus tectorum* seed banks in semi-arid western North America, exhibits >4-fold variation in mycelial growth rate. Host seeds exhibit seasonal changes in dormancy that affect the risk of pathogen-caused mortality. The hypothesis tested is that contrasting seed dormancy phenotypes select for contrasting strategies for increasing pathogen fitness, and that increased fitness on nondormant seeds involves a resource trade-off between toxin production and growth. The strategy for successfully attacking rapidly germinating nondormant seeds at high inoculum loads in autumn involves increased post-infection aggressiveness to prevent seed escape through germination. An earlier study demonstrated that slow-growing strains caused higher mortality than faster-growing strains on nondormant host seeds at high inoculum loads. In this study, production of the toxin cytochalasin B was significantly higher in slower-growing strains, and was induced only in seeds or in seed-constituent-containing media. Its production was reduced *in vivo* by *Bromus tectorum* seeds, suggesting direct involvement in pathogenesis on seeds. Fast-growing strains caused significantly higher mortality than slow-growing strains at low inoculum loads on dormant seeds, which apparently have resistance that is overcome at high loads or through rapid mycelial proliferation. In a co-inoculation study, the fast-growing isolate produced 3 × more stromata than the slow-growing isolate on dormant seeds, whereas the slow-growing isolate was twice as successful on nondormant seeds. These results provide evidence that mycelial growth rate variation and associated variation in cytochalasin B production represent a trade-off maintained through temporally varying selection resulting from seasonal variation in host seed dormancy status.

Keywords: *Bromus tectorum*, co-inoculation, competition, cytochalasin B, seed bank, seed dormancy

Introduction

Pathogenicity, also referred to as infectivity, is the ability of a pathogen to infect, grow, and reproduce in a host organism. In a recent review, Tack *et al.* (2012) demonstrated that spatiotemporal variation in pathogenicity, both within and among populations, is ubiquitous across a wide range of pathosystems. Because increased pathogenicity is predicted to be associated with increased fitness and therefore to be under positive selection, variation in pathogenicity must be maintained through more complex evolutionary processes. For example, variation in pathogenicity could be maintained through frequency-dependent selection (Leonard, 1997) or through trade-offs with other aspects of pathogen life history (Laine & Barrès, 2013). Both theoretical and empirical studies of spatiotemporal variation in pathogenicity in plant pathosystems have most often been carried out

using foliar pathogens with at least some degree of host genotype specialization and with efficient aerial spore dispersal. Tack *et al.* (2012) noted that there is a paucity of information both on generalist plant pathogens and on soilborne pathogens with limited dispersal ability. They emphasized that studies of such pathogens would greatly increase the power of generalization about the underlying causes of spatiotemporal variation in pathogenicity. Specifically, soilborne seed pathogens that attack the post-dispersal stage of the plant life cycle remain poorly studied, even though they are potentially of major ecological significance in natural systems (Gilbert, 2002).

Seed pathogens differ fundamentally from the parasites considered in classical models of the evolution of virulence (Meyer *et al.*, 2010). For pathogens or parasites that cause disease on actively growing organisms, excessive virulence or aggressiveness (i.e. ability to inflict damage) resulting in premature host death can theoretically reduce pathogen reproduction and transmission, thereby lowering fitness (Anderson & May, 1982; Alizon *et al.*, 2009). Seed pathogens may be considered to behave more like predators than parasites. Because a seed represents a finite resource, the ability to cause host death increases pathogen fitness by eliminating the possibility

*E-mail: smeyer@fs.fed.us

that the seed will germinate successfully and usurp this finite resource for its own growth. For seed pathogens that cannot subsequently impact survival of seedlings, seed escape through germination can be regarded as analogous to prey escape in a predator–prey system (Beckstead *et al.*, 2007). Pathogen fitness is reduced on infected seeds that successfully germinate because, even though post-germination sporulation is sometimes possible, pathogen reproductive output is reduced relative to reproductive output on killed seeds.

Most studies of frequency-dependent selection and other forms of temporally varying selection in plant pathosystems have focused on changes in the relative abundance of different races or pathotypes within a pathogen population, i.e. subsets of the pathogen population that specialize to at least some degree on different host genotypes (Sacristan & García-Arenal, 2008). However, temporally varying selection could also operate in pathosystems that completely lack host genotype specialization (Barrett *et al.*, 2009). For example, if different developmental phenotypes of the same host genotype favoured the success of different pathogen genotypes within a population, then genetic variation in pathogen traits mediating this differential success could be maintained (Pfennig, 2001). Also, differences in success on different host developmental phenotypes could involve resource trade-offs, such that the pathogen genotype most successful on one host phenotype would be at a selective disadvantage on the contrasting host phenotype. This form of selection could operate in the absence of genetic variation in host resistance.

Life history trade-offs were first proposed in the context of host–parasite relationships (Anderson & May, 1982), but the concept has been explored in studies of many plant pathosystems. Many studies have addressed the idea of a cost of excessive virulence, i.e. the cost of maintaining virulence genes with no corresponding resistance genes in the co-occurring host population (Frank, 1992). Other studies examine trade-offs between within-season fitness (pathogenicity, spore production) and between-season fitness (survival; Andrivon *et al.*, 2013), or between latent period (analogous to age at reproduction) and spore production or transmission success (equivalent to fecundity; Héraudet *et al.*, 2008; Pariaud *et al.*, 2013). These studies sometimes include a host phenotypic variation component, which can affect the balance point of the trade-off, so that on some host phenotypes, for example, latent period is reduced at the expense of spore production, whilst on others, spore production is increased but latent periods are longer (Pariaud *et al.*, 2013). One potential effect of life history trade-offs in plant pathosystems is to increase genetic variation in the pathogen population, as there is often no single optimal solution to the problem of metabolic resource allocation.

In the study reported here, the effects of temporally varying selection and resource trade-offs on maintenance of genetic variation in mycelial growth rate (MGR) for a generalist seed pathogen are examined. The necrotrophic

seed pathogen *Pyrenophora semeniperda* is important in seed banks of the winter annual grass *Bromus tectorum* in semi-arid western North America (Meyer *et al.*, 2007). In the *B. tectorum* seed banks where *P. semeniperda* is abundant, seeds pass through a continuum of dormancy states (Meyer & Allen, 2009; Allen *et al.*, 2010). At dispersal in early summer, the seeds are in a state of primary dormancy and germinate slowly if at all, even under optimal conditions. This dormancy is gradually lost through the summer months through dry after-ripening, so that when the first germination-triggering rains of autumn arrive, the nondormant seeds are poised to germinate very quickly. Adequate autumn rainfall often results in near-complete germination, but if rainfall is sporadic or lacking, a sizeable fraction of the current-year seed will remain ungerminated. As temperatures grow cooler, these ungerminated seeds cycle back into dormancy, which may again be lost through dry after-ripening the following summer (Allen *et al.*, 2010). This pattern of dormancy loss and induction means that there is seasonal variation in the physiological status of seeds that are subject to pathogen attack (Fig. 1).

Pyrenophora semeniperda inoculum load also varies seasonally in the field in this pathosystem (Fig. 1; S. E. Meyer, unpublished data). Most sporulation takes place in late spring, so that high loads are present through the summer and during the first autumn rains. Pathogen conidia germinate in response to these rains along with most host seeds, and the inoculum reservoir is depleted. When temperatures warm in spring and host seeds are in secondary dormancy and subject to pathogen attack, inoculum loads remain low, even though many seeds are infected, because sporulation in this dry-sporing fungus does not take place until the seed bed begins to dry. Thus high inoculum loads are present when seeds are nondormant in autumn, but inoculum loads are much lower when seeds are dormant in early spring prior to soil drying.

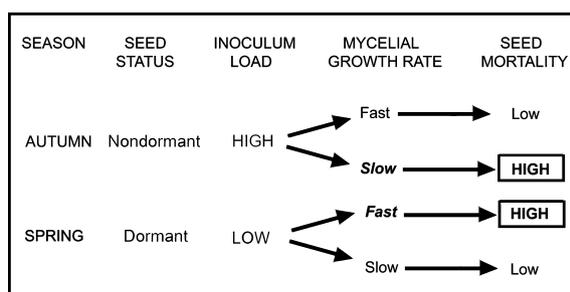


Figure 1 Schematic diagram of predicted relationships among season, seed dormancy status, inoculum load and strain mycelial growth rate in terms of effect on seed mortality level, an indirect measure of pathogen fitness. In autumn, seeds are nondormant and inoculum loads are high, leading to higher seed mortality and therefore increased fitness for slow-growing strains. In spring, seeds are dormant and inoculum loads are low, leading to higher seed mortality and therefore increased fitness for fast-growing strains.

Pyrenophora semeniperda exhibits wide among-isolate variation in MGR in solid culture (Meyer *et al.*, 2010). In an earlier study with *B. tectorum* host seeds, germination speed was the main factor determining mortality levels for host seeds, with faster-germinating seeds more likely to escape *P. semeniperda*-caused mortality even if infected (Beckstead *et al.*, 2007, 2014). Seeds that develop pathogen stromata prior to germination are killed, whereas infected seeds that germinate may develop normal seedlings even though some pathogen sporulation subsequently occurs. The pattern of higher survival for faster-germinating host seeds represents a form of tolerance that results in minimal damage subsequent to pathogen infection (Roy & Kirchner, 2000). This ‘race for survival’ on the part of host seeds was hypothesized to drive positive selection for faster-growing pathogen strains. However, when the relationship between MGR and the ability to cause seed mortality at high inoculum loads on nondormant *B. tectorum* seeds was examined experimentally, the opposite pattern was observed (Meyer *et al.*, 2010). Slower-growing pathogen strains caused significantly more seed mortality under this scenario than faster-growing strains. The proposed explanation for this counter-intuitive result was that the ability to cause mortality of nondormant seeds was associated with production of a metabolically expensive toxin that could disable germinating seeds, in effect creating a resource trade-off between MGR and the high aggressiveness on nondormant seeds associated with high toxin production.

The hypothesis tested here is that contrasting host seed dormancy phenotypes select for contrasting strategies for increasing pathogen fitness (Fig. 1; Pfennig, 2001). The strategy for successfully attacking rapidly germinating nondormant seeds at relatively high inoculum loads in the autumn seed bank is hypothesized to include the MGR–toxin production trade-off explained earlier, which involves increased aggressiveness post-infection to maximize fitness by preventing escape through germination. This is postulated to cause the correlation observed between slower MGR and increased nondormant seed mortality (Meyer *et al.*, 2010). The pathogen strategy on dormant seeds does not need to include high aggressiveness, as once dormant seeds are infected, they are not capable of escaping through germination. However, dormant *B. tectorum* seeds are known to possess general resistance to pathogen infection at low inoculum loads, and the degree of resistance varies among host genotypes (Meyer & Clement, 2014). This resistance can be overcome at high inoculum loads in the laboratory, which generally result in near-complete mortality of dormant seeds regardless of host or pathogen genotype. At the low loads characteristic of winter/spring seed banks, the probability of seed survival is hypothesized to depend on pathogen as well as host genotype. Under this scenario, fast-growing strains would exhibit increased pathogenicity, i.e. increased ability to infect, grow and reproduce inside the host, because rapid mycelial proliferation inside the seed would tend to overcome resistance in

much the same way as high inoculum load. The evolutionary outcome of these contrasting strategies would be temporally varying selection on MGR that reflects this seasonal variation in seed dormancy status (Fig. 1).

In order to test the MGR–toxin production trade-off hypothesis, a series of studies building on earlier work (Evidente *et al.*, 2002) was first conducted to characterize and quantify secondary metabolite production in this pathogen (Masi *et al.*, 2014a,b,c,d). The principal toxic metabolite produced by the fungus is cytochalasin B, a compound well known for its ability to disrupt cell division through its effects on the actin skeleton during cytokinesis (Möbius & Hertweck, 2009; Scherlach *et al.*, 2010). *Pyrenophora semeniperda* is known to produce cytochalasin B in large quantities (up to 2300 mg kg⁻¹) in standard autoclaved wheat seed culture, but it does not produce this compound at all in liquid culture in Fries medium or potato dextrose broth (Evidente *et al.*, 2002; Masi *et al.*, 2014a).

The research described here focuses on cytochalasin B as the putative seed-disabling toxin that enables slow-growing pathogen strains to kill nondormant seeds. The study had the following objectives: (i) determine the range and distribution of temporal and local spatial variation in MGR in *P. semeniperda*; (ii) determine whether *P. semeniperda* produces cytochalasin B in *B. tectorum* seeds, either *in vitro* in autoclaved seeds or *in vivo*; (iii) establish the conditions under which cytochalasin B production is induced in liquid culture; (iv) determine whether there is a negative relationship (i.e. a trade-off) between MGR and cytochalasin B production; (v) determine whether there is a positive relationship between MGR and dormant seed mortality at low inoculum loads; and (vi) determine whether the competitive outcome of co-inoculation with fast- and slow-growing strains of the pathogen in terms of reproductive success (stromatal production) differs on dormant versus nondormant seeds.

Materials and methods

Experiment 1. Mycelial growth rate (MGR) variation among and within populations

Initial MGR measurements in solid culture on potato dextrose agar (PDA) were made for a total of 100 isolates obtained from the Tenmile Creek, UT, USA (41.8649°N, 113.136°E, 1453 m a.s.l.), the Whiterocks Exlosure, UT (40.328°N, 112.778°E, 1446 m a.s.l.), and the Whiterocks Road, UT (40.329°N, 112.889°E, 1567 m a.s.l.) populations in 2010, and for 72 isolates obtained from the Tenmile Creek and Whiterocks Exlosure populations in 2006.

For determination of MGR in solid culture, single conidia of each isolate were transferred to the centre of 100 mm Petri dishes containing quarter-strength PDA and incubated at 20°C for 14 days (see Meyer *et al.*, 2010 for details). Radial growth along four axes at 45° angles was measured, and the four colony diameter measurements were averaged for each Petri dish. Two dishes were prepared for each isolate. Mean radial growth rate per day (mm per day) was obtained by dividing the mean colony diameter by 14 (days).

These data were summarized in frequency histograms for each population and year in order to display the range of variation in MGR within and among populations. Frequency distributions based on the raw count data were compared using Fisher's exact tests for small cell values to evaluate among-year and among-population differences.

Ten isolates from the 2010 isolate collection were selected to represent a range of growth rates and were used for the study reported in Masi *et al.* (2014a) and also for experiments reported here for pathogenicity on dormant seeds at low inoculum loads. The experiment with conidial inoculum on dormant seeds included an additional 2010 isolate for a total of 11. The 2010 isolate collection was also used to obtain 20 additional isolates with a range of growth rates for the experiment on cytochalasin B production in liquid culture. These 20 isolates were re-evaluated on PDA and also on oatmeal agar, using the same MGR measurement protocol with two replicates per isolate.

Experiment 2. Cytochalasin B induction in solid and liquid culture

To determine cytochalasin B production in solid culture on autoclaved and living (dormant) host (*B. tectorum*) seeds, 200 g of cleaned seeds (florets with lemma and palea still attached to the caryopses) were soaked in sterile water, inoculated with 6.6 mg of isolate WRK10-22 conidial inoculum suspended in sterile water, and incubated at room temperature (*c.* 22°C) for 4 weeks in a 1 L Erlenmeyer flask with a loose aluminum cap (standard or wet condition) or a flat aluminum pan with a plastic cover (drier condition). The flask was shaken periodically to prevent the seeds from caking together. After 4 weeks the solid seed cultures were spread in aluminum pans to air dry. The air-dried cultures were then ground and extracted, and cytochalasin B content was determined (Data S1).

The goal of the liquid culture experiments was to find a medium that would induce cytochalasin B production to use in the growth rate experiment. The working hypothesis was that seed constituents included in the liquid medium would tend to induce cytochalasin B production. These experiments were carried out in three phases. In the first phase, complex media (broths) were tested: potato dextrose broth (PDB), modified alphacel medium (MAM; coconut milk–oatmeal), coconut milk–oat flour, coconut milk–*B. tectorum* flour, V-8, tomato paste–oat flour, tomato paste–wheat flour and tomato paste–*B. tectorum* flour. In the second phase, individual ingredients from these complex media were tested singly: oatmeal, oat flour, tomato paste, and coconut milk. In the third phase, a reliable inducing medium (oatmeal broth) was modified by using an oatmeal infusion at two concentrations instead of solid oatmeal, with or without the addition of mineral nutrients and dextrose. To produce the oatmeal infusion medium for each experimental unit, either 3.75 g or 7.5 g of oatmeal (low concentration and high concentration, respectively) were added to 125 mL DI (deionized) water enclosed in a stapled cheesecloth bag and autoclaved at 100°C for 60 min to cook; the bag was then squeezed slightly to press out excess liquid. The liquid was brought back up to 125 mL volume, and 0.125 g magnesium sulphate heptahydrate, 0.1875 g monopotassium phosphate, 0.125 g sodium nitrate and 2.5 g dextrose were added for the plus nutrients treatment. The mixture in each flask was then autoclaved at 121°C for 15 min. The objective of this phase was to identify a liquid medium that would permit mycelial growth, induce cytochalasin B production, and allow accurate measurement of mycelial biomass without interference from solids suspended in the medium.

Each medium tested (125 mL) was inoculated with an actively growing mycelial plug of isolate WRK10-22 and incubated in shaker culture (150 rpm at 22°C for 14 days) in a 200 mL Erlenmeyer flask with a sterile cotton stopper. Each culture was then filtered using a 0.45 µm CN membrane filter unit (Fisher Scientific), and the filtrate was extracted for cytochalasin B quantification (Data S1). These experiments were not replicated, except for instrumental technical replication during quantification ($n = 3$).

Experiment 3. MGR × cytochalasin B production

Mycelial growth rate and cytochalasin B production were measured in the same experimental system in liquid culture for the 20 *P. semeniperda* isolates selected from the 2010 collection to represent a range of MGRs in solid culture. For each experimental unit, a 3 mm plug of actively growing mycelium from the edge of a 5-day-old solid oatmeal culture was added to 125 mL of high-concentration oatmeal infusion with added dextrose and mineral nutrients and incubated in shaker culture as described above for 10 days. This incubation period was chosen on the basis of preliminary data showing that cytochalasin B production had occurred by this time but mycelial biomass had not yet peaked even for faster-growing isolates. The experiment was carried out in three temporal blocks (repeats). Each experimental repeat consisted of one flask of each of the 20 isolates. At the end of the incubation period, the contents of each flask were filtered from the medium as described earlier, dried on pre-tared filter paper at 35°C, and weighed. The filtrate was retained for extraction and cytochalasin B quantification (Data S1). This permitted expression of cytochalasin B production as mg mg⁻¹ of mycelial biomass.

The resulting data set was subjected to mixed model analysis of covariance (SAS Institute, PROC MIXED) with experimental repeat as the random categorical variable and mycelial growth rate (MGR) as the continuous variable using all the data points ($n = 60$). The response variable was cytochalasin B production per unit of mycelial biomass, while the predictor variable (MGR) was expressed either as 10-day mycelial biomass in oatmeal infusion culture for each experimental replicate or as mean mm per day in oatmeal agar culture for each isolate. Data are presented in the regression plot as isolate means with standard errors for cytochalasin B production.

Experiment 4. MGR and virulence on dormant seeds

To measure differences in the ability of isolates to cause dormant seed mortality at a low inoculum load, conidia of each of the 11 isolates from 2010 chosen to represent a range of MGR were checked for conidial germination, and prepared as diluted inoculum at a ratio of 1:3200 germinable conidia. Talc, an inert powder shown earlier to have no effect on either conidial germination or seeds, was used as the diluent (Beckstead *et al.*, 2014). This dilution yielded approximately 50% mortality on dormant seeds in previous experiments, enabling the detection of among-isolate differences in seed mortality. The experiment was carried out in two temporal blocks (repeats). Each repeat included eight experimental units for each isolate and for the uninoculated controls, which exhibited no seed mortality (data not shown). An experimental unit consisted of 50 dormant seeds (greenhouse-produced seeds grown from a bulk collection in 2008 from the Whiterocks Exclusion) inoculated by vortexing 3 mg of diluted inoculum with the seeds in a small vial. The seeds were then spread on the surface of two water-soaked germination blotters

in a 100 mm Petri dish. The dishes were incubated at 20°C for 2 weeks to allow time for pathogen stromatal development on the seeds, and the number of ungerminated seeds with pathogen stromata in each dish was determined. As mentioned earlier, ungerminated seeds that exhibit stromatal development are known to be killed (Beckstead *et al.*, 2007). The data set was subjected to mixed model analysis of covariance with mycelial growth rate as the continuous effect, experimental repeat as the random categorical effect, and arcsine-square root transformed killed seed proportion as the dependent variable. The regression is presented using untransformed isolate means with standard errors for seed mortality.

A second method was also used to measure differences in the ability of the 10 isolates described earlier to cause seed mortality at relatively low inoculum loads. This method used bulk inoculum produced using a protocol originally developed for field application. The inoculum was produced by growing each isolate in shaker culture in PDB (14 days at 150 rpm at 22°C), centrifuging, discarding spent medium, then adding the mycelial material to an inert carrier (vermiculite) along with MAM as a supplemental nutrient source. After thorough mixing, the inoculum was spread in trays and allowed to dry slowly under cool-white fluorescent and UV light, conditions known to stimulate sporulation, for 48 h (Campbell *et al.*, 2003). It was then transferred to a warm greenhouse (*c.* 30°C) to complete drying. The dry inoculum was then hand-crushed and weighed for application.

The experiment consisted of a randomized block design with four blocks. The experimental units were seed bed microcosms obtained by taking soil surface cores from *B. tectorum* monocultures with 9 cm diameter steel rings cut in 2 cm increments from muffler pipe (see Beckstead *et al.*, 2010 for details). These seed bed microcosms were autoclaved intact to provide a planting medium that simulated field physical conditions but that contained no viable pathogen propagules or seeds. The bulk inoculum was diluted 1:10 with additional vermiculite, then added to each ring microcosm by sprinkling 0.23 g of diluted inoculum onto the surface. For each experimental unit, 50 dormant *B. tectorum* seeds, dyed pink with nontoxic safranin for ease of recovery, were planted into the soil surface. The microcosms were kept moist under shade cloth in a cool greenhouse (20–25°C) for 4 weeks. Planted seeds were then recovered by hand from each microcosm and examined for germination and/or pathogen stromata. Ungerminated seeds with stromata were scored as killed. Remaining ungerminated seeds were placed on germination blotters in Petri dishes and incubated for 7 additional days to allow time for pre-infected seeds to develop disease signs. The number of killed seeds (ungerminated seeds with stromata) and the total number of remaining seeds were then determined. The killed seed proportion in each experimental unit was calculated as (number killed in microcosm + number killed in post-recovery incubation)/total seeds recovered. Recovery averaged 48 of 50 planted seeds.

The data set was analysed using mixed model analysis of covariance with block as the categorical random effect, MGR as the continuous effect, and arcsine-square root transformed mortality proportion as the dependent variable. The regression is presented using untransformed isolate means with standard errors for seed mortality.

Experiment 5. Co-inoculation on dormant versus nondormant seeds

To test the hypothesis that isolates with contrasting growth rates would show differential success when co-inoculated onto dor-

mant versus nondormant seeds, two isolates were chosen from the set used in the earlier experiment relating MGR to virulence on nondormant seeds (Meyer *et al.*, 2010). TMC23 (collected at Tenmile Creek, UT in 2006) was the most aggressive isolate in the earlier study and one of the slowest growing, while MLV07 (collected from Middle Love Valley, Turkey in 2009; Stewart *et al.*, 2009) was one of the least aggressive and fastest-growing. These two isolates had contrasting genotypes at both the ITS locus (Boose *et al.*, 2011) and at three of seven microsatellite markers that had been developed from genome sequence data for this organism (Soliai *et al.*, 2014). This made it possible to genotype individual stromata from killed seeds resulting from the co-inoculation and to determine the relative number of stromata produced by each isolate on dormant versus nondormant seeds. For genotyping, one or two stromata from individual killed seeds in each co-inoculation treatment were removed and each stroma was subjected to DNA extraction and microsatellite fingerprinting (Data S2).

The co-inoculation experiment was carried out using nondormant and dormant *B. tectorum* seeds collected at Saddle Mountain, WA in 2008 (nondormant) or 2009 (dormant). Seeds were first surface-sterilized via submersion for 1 min each in 70% ethanol, 10% bleach, and DI water. Inoculation treatments consisted of an uninoculated control, a single inoculation for each isolate of *P. semeniperda*, and a double inoculation with both isolates. Surface-sterilized seeds were inoculated by submersion in a conidial suspension followed by shaking at 300 rpm for 1 h. Control seeds were inoculated with a mock suspension of DI water and exhibited no seed mortality (data not shown). Single inoculations consisted of suspensions of 5000 conidia mL⁻¹. The double inoculation contained 2500 conidia mL⁻¹ of each isolate. This method resulted in a single relatively high inoculum load; inoculum load was not manipulated in this test. An experimental unit consisted of 25 seeds appropriately inoculated and placed on germination blotters in a 100 mm plastic Petri dish. Dishes were incubated in the dark at 20°C for approximately 4 weeks. A seed was considered killed if it both failed to germinate and displayed fungal stromata protruding from the seed coat. Remaining seeds were scored as germinated or subjected to a cut test to evaluate viability (Ooi *et al.*, 2004). In each experiment, each set of treatments was replicated 10 times, and the experiment was carried out in two temporal blocks (repeats). For mixed model analysis of variance (SAS Institute, PROC MIXED), dormancy status and inoculation treatment were considered fixed effects and repeat was considered the random effect. The response variable was arcsine-square root-transformed killed seed proportion. Least squares means separation tests were used to compare the three inoculation treatments for dormant and nondormant seeds.

Results

Experiment 1. MGR variation among and within populations

Mycelial radial growth rate as measured on PDB at 20°C varied widely among isolates within populations, and ranged from 1.27 to 5.14 mm per day (Fig. 2). This difference is even more dramatic when expressed as areal growth rate (range 5–83 mm² per day), which increases exponentially with radial growth rate and relates more directly to differences in mycelial biomass. The relative frequency of isolates in different growth rate categories

varied among years in both the Tenmile Creek and Whiterocks Exclosure populations, with a significant shift to more slow-growing isolates in 2010 vs. 2006 in both populations ($P = 0.0105$ and $P = 0.0007$, respectively; Fisher's exact test). In 2010 the population at the Whiterocks Road study site appeared to have a somewhat wider spread of growth rates than the Whiterocks Exclosure population 9 km away, where growth rates were concentrated in the intermediate categories, but the two distributions were not significantly different ($P = 0.166$). These results show that the relative abundance of fast- and slow-growing isolates can shift

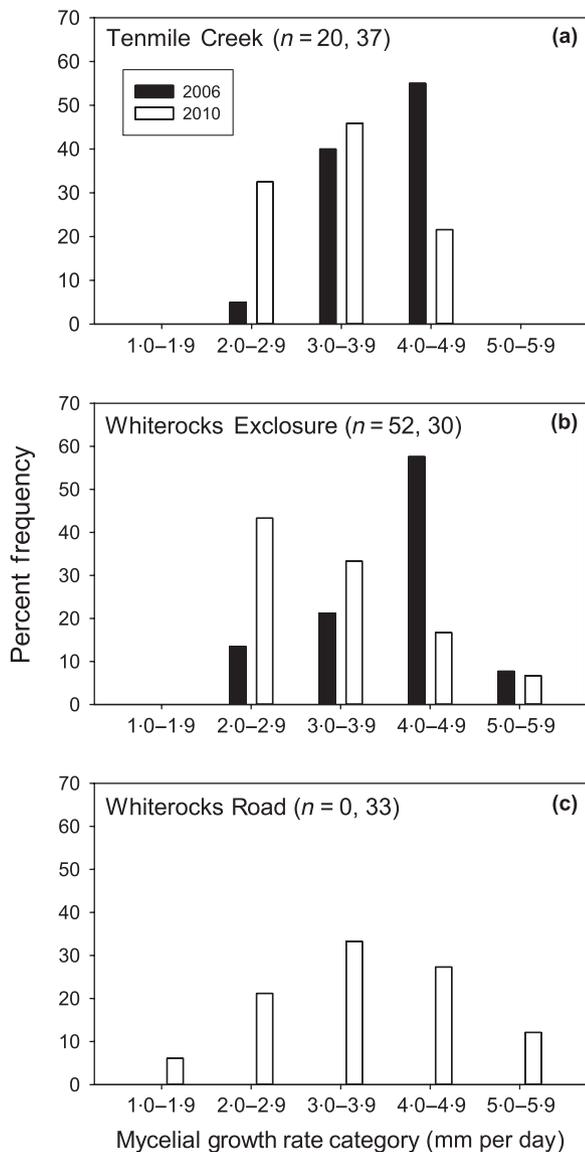


Figure 2 Temporal and local spatial variation in the distribution of mycelial growth rates among isolates in three study populations: (a) Tenmile Creek sampled in 2006 and 2010, (b) Whiterocks Exclosure sampled in 2006 and 2010, and (c) Whiterocks Road (9 km from Whiterocks Exclosure) sampled in 2010.

through time and can also vary on a local spatial scale, but that a mixture of fast- and slow-growing isolates is generally present. This general pattern of high MGR variation has been observed for several additional populations from throughout the region (data not shown).

Experiment 2. Cytochalasin B production in solid and liquid culture

Cytochalasin B was produced in large quantities by *P. semeniperda* isolate WRK10-22 in solid culture on *B. tectorum* seeds under all cultural conditions (Fig. 3a). The production on autoclaved *B. tectorum* seeds under standard conditions was essentially identical to the pro-

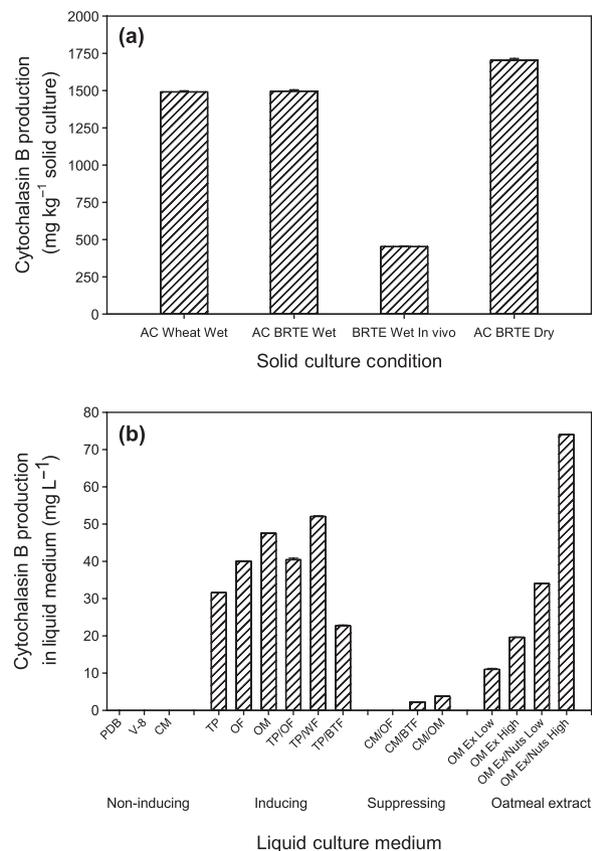


Figure 3 Cytochalasin B production by *Pyrenophora semeniperda* isolate WRK10-22 as a function of cultural conditions: (a) After 28 days at c. 22°C in solid culture on autoclaved wheat seeds under standard (wet) conditions (AC wheat wet; data from Masi *et al.*, 2014a), on autoclaved *Bromus tectorum* seeds under standard (wet) conditions (AC BRTE wet), *in vivo* on dormant host (BRTE) seeds under standard (wet) conditions (BRTE wet *in vivo*), and on autoclaved host (BRTE) seeds under drier conditions (AC BRTE dry); (b) After 14 days at c. 22°C in liquid media that were cytochalasin B non-inducing, cytochalasin B inducing, or cytochalasin B suppressing (PDB, potato dextrose broth; V-8, V-8 broth; CM, coconut milk; TP, tomato paste; OF, oat flour; OM, oatmeal; WF, wheat flour; BTF, host seed flour). The last four bars show cytochalasin B production in oatmeal extract broth (OM EX) at two strengths, with and without added mineral nutrients and dextrose. Error bars = SE (from technical replication, $n = 3$).

duction for this same isolate in autoclaved wheat seed culture under the same conditions as reported earlier (Masi *et al.*, 2014a). Production increased 14% when autoclaved *B. tectorum* seeds were incubated under slightly drier conditions more conducive to growth for this pathogen. However, when the pathogen was inoculated onto dormant *B. tectorum* seeds and allowed to develop *in vivo* under standard conditions, cytochalasin B production was reduced by a factor of three, from 1496 mg kg⁻¹ on autoclaved seeds to 492 mg kg⁻¹ on dormant seeds. This indicates that living dormant host seeds have some capacity to suppress cytochalasin B production.

It was possible to induce cytochalasin B production by *P. semeniperda* in liquid culture by adding seed constituents (other than from non-host coconut seeds) to the liquid medium (Fig. 3b). Media that did not contain host seed constituents (PDB, V8, coconut milk) did not induce production, whereas media that contained oatmeal, oat flour, wheat flour, *B. tectorum* flour or tomato paste were cytochalasin B-inducing. Media that contained coconut milk in addition to one of the cytochalasin B-inducing constituents resulted in the production of little or no cytochalasin B, indicating that coconut milk could suppress cytochalasin B production even in the presence of inducing elements.

Oatmeal infusion at the high level with addition of dextrose and mineral nutrients induced the highest production of cytochalasin B (Fig. 3b) and was therefore chosen as the medium for the growth rate × cytochalasin B production study.

Experiment 3. MGR × cytochalasin B production

Cytochalasin B production per unit of mycelial biomass in liquid culture at 22°C was generally high but varied significantly among isolates ($F = 9.68$, d.f. = 19, 38, $P < 0.0001$). Mean values ranged from 0.0038 to 0.0132 mg mg⁻¹, a 3.5-fold variation. In contrast, total biomass varied only over a 1.4-fold range (0.444–0.642 g dry biomass 100 mL⁻¹), although differences among isolates were still significant ($F = 2.57$, d.f. = 19, 38, $P = 0.0065$). Because of the rather uniform mycelial biomass yields, total cytochalasin B production was significantly correlated with production on a unit biomass basis ($r = +0.900$, d.f. = 58, $P < 0.0001$).

Contrary to the predicted outcome, there was no correlation between mycelial biomass in liquid culture (a measure of MGR) and cytochalasin B production per unit of mycelial biomass ($r = -0.048$, d.f. = 58, $P = 0.7157$). This was probably because MGR under the conditions of liquid culture was largely invariant, as mentioned above. Mycelial biomass in liquid culture was also not at all correlated with MGR in the oatmeal agar culture used to choose isolates with a range of growth rates ($r = 0$, d.f. = 58, $P = 0.9289$). This suggests that the liquid cultures were produced under growth-limiting conditions that did not permit the expression of meaningful differences in growth rate, possibly because the

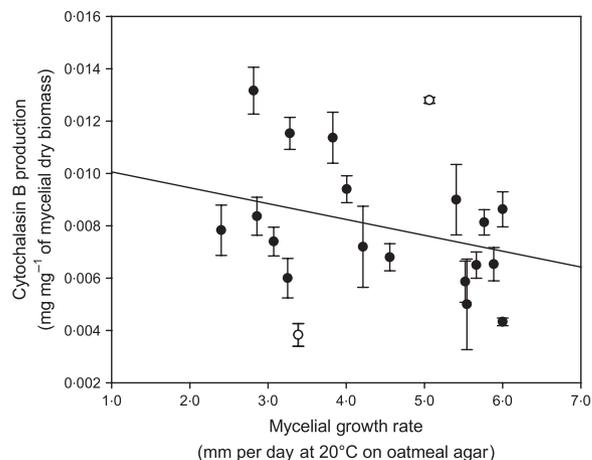


Figure 4 The negative relationship between mycelial growth rate (MGR) in solid culture on oatmeal agar and production of cytochalasin B per unit of mycelial dry biomass in oatmeal liquid culture for 20 *Pyrenophora semeniperda* isolates. The liquid culture experiment included three temporal blocks. Analysis of covariance was performed with temporal block as the random categorical variable and MGR on oatmeal agar as the continuous variable (d.f. = 1, 58, $F = 4.20$, $P = 0.045$). Data are shown as isolate means with error bars (SE) for cytochalasin B production. Apparent biological outliers (white symbols) were retained in the analysis.

oatmeal infusion did not provide sufficient carbohydrate as a substrate for growth, even though conditions for cytochalasin B induction were met.

When cytochalasin B production per unit of mycelial biomass in liquid culture was plotted against MGR in the oatmeal agar plates used to produce the liquid cultures, analysis of covariance showed that the relationship was significant and had the predicted negative slope (Fig. 4). It was necessary to use mean values of MGR for this analysis because MGR and cytochalasin B production were not measured simultaneously in the same experimental units as they had been in the liquid culture experiment. Although the variance accounted for was low, this analysis provided support for the hypothesis that MGR and cytochalasin B production per unit biomass are negatively correlated. The relatively poor fit could be due to the fact that the conditions in oatmeal agar were considerably different than conditions in growth-limiting liquid culture. It is remarkable that cytochalasin B production per unit biomass was significantly correlated with growth rate in solid culture even under these growth-limiting conditions. Two isolates that appear to be biological outliers appear as open circles on the plot, but their replicate values are included in the analysis.

Experiment 4. MGR and virulence on dormant seeds

When the isolates included in Masi *et al.* (2014a) were tested for pathogenicity at low inoculum loads on dormant seeds in two independent tests using different methods, highly significant positive relationships between

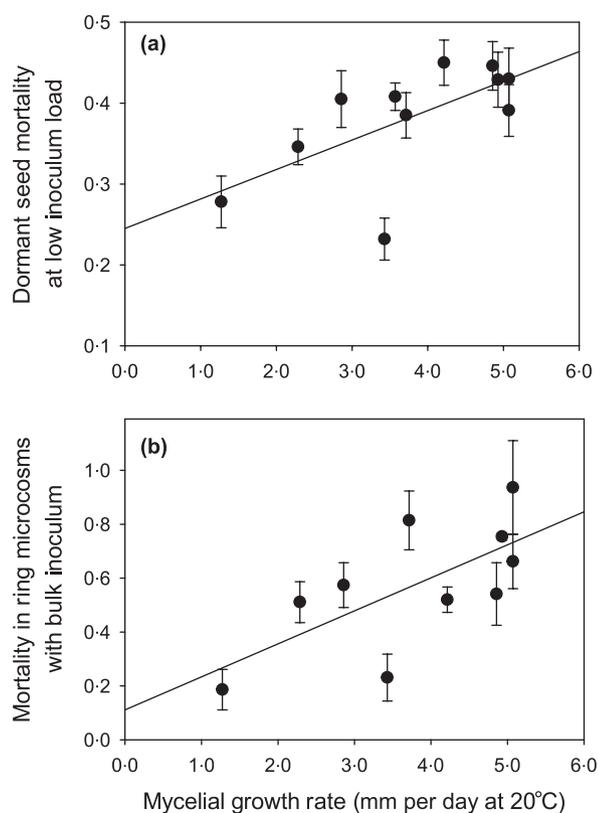


Figure 5 The positive relationship between mycelial growth rate (MGR) on quarter-strength potato dextrose agar (PDA) and (a) dormant seed mortality at low conidial inoculum load (d.f. = 1,173, $F = 20.73$, $P < 0.0001$), (b) dormant seed mortality in ring microcosms with bulk inoculum (d.f. = 1, 35, $F = 14.53$, $P = 0.0005$). Data were analysed using mixed model ANCOVA with block as the random variable and MGR on PDA as the continuous variable and are presented as isolate means with error bars (SE) for seed mortality [$n = 16$ replicates for (a), $n = 4$ replicates for (b)].

MGR and mortality of dormant seeds were obtained in both cases, supporting the hypothesis that faster-growing isolates are more effective at causing dormant seed mortality (Fig. 5a,b). Mean seed mortality levels in the two dormant seed experiments were also significantly positively correlated with each other ($r = +0.792$, d.f. = 8, $P = 0.0063$), but were not significantly negatively correlated with cytochalasin B production in solid autoclaved wheat seed culture as reported in Masi *et al.* (2014a).

Experiment 5. Co-inoculation on dormant vs. nondormant seeds

When fast-growing and slow-growing isolates were inoculated singly or in combination onto dormant seeds, there were no significant differences among the three treatments in seed mortality, as expected at the relatively high inoculum load used in this experiment (Fig. 6a). Mean mortality on dormant seeds was 84%. On nondormant seeds, the fast-growing isolate caused 24% seed mortality and the slow-growing isolate caused 37% seed

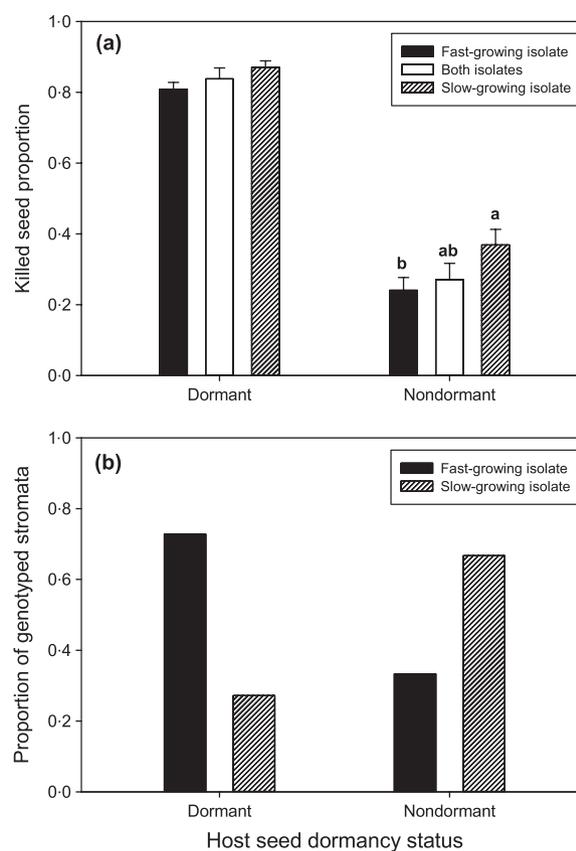


Figure 6 (a) Proportion of host seeds killed by a fast-growing isolate, a slow-growing isolate, and in a co-inoculation with both isolates on dormant and nondormant host seeds. Isolate means for nondormant seeds headed by different letters are significantly different according to LSmeans separation from analysis of variance ($P = 0.0268$); (b) proportion of stromata of each of two isolates recovered from killed dormant vs. nondormant host seeds that had been co-inoculated with conidia of fast-growing and slow-growing isolates with contrasting microsatellite genotypes ($\chi^2 = 11.797$, d.f. = 1, $P = 0.0006$; $n = 162$ dormant seeds, $n = 18$ nondormant seeds).

mortality, while the co-inoculation with both isolates resulted in intermediate mortality (27%). The difference in seed mortality between the fast-growing and slow-growing isolates was significant ($P = 0.0268$).

When individual stromata from killed seeds in the dormant seed co-inoculation treatment were genotyped, 118 of 162 stromata were identified as belonging to the fast-growing isolate, while the remainder belonged to the slow-growing isolate (Fig. 6b). Many fewer stromata were obtained from nondormant seeds because of the much lower mortality rate, but of the 18 stromata genotyped, 12 belonged to the slow-growing isolate and only six belonged to the fast-growing isolate. Contingency table analysis demonstrated that these two frequency distributions were significantly different ($\chi^2 = 11.797$, d.f. = 1, $P = 0.0006$). These results support the hypothesis that fast-growing strains will be more successful on dormant seeds in competition with slow-growing strains,

while on nondormant seeds, slow-growing strains will be more successful. This indicates that in mixed-strain infections, the fitness outcomes for fast- vs. slow-growing strains in competition may depend on the dormancy status of the host seed.

Discussion

The results of the research presented here provide evidence that the wide variation in MGR consistently observed in wild populations of the seed pathogen *P. semeniperda* on *B. tectorum* (Fig. 2) is maintained at least in part as a result of selection for contrasting MGR on nondormant vs. dormant seeds in the seed bank. Fast mycelial growth was demonstrated in two independent experiments to be associated with increased pathogenicity (infectivity) on dormant seeds at the low inoculum loads probably encountered in winter/spring seed banks (Figs 1 & 5a,b). Dormant seeds possess resistance to infection that can apparently be more readily overcome by faster-growing strains, perhaps because rapid mycelial proliferation within the seed mimics the effect of high inoculum load. In contrast, slow mycelial growth is associated with increased aggressiveness on nondormant seeds at the relatively high inoculum loads in autumn seed banks (Meyer *et al.*, 2010). Most nondormant seeds may become infected under these conditions, but they are relatively tolerant to infection and capable of rapid germination, so that pathogen fitness is maximized only by high post-infection aggressiveness that can prevent escape through germination, resulting in seed mortality.

This study also showed that the difference in post-infection aggressiveness between fast- and slow-growing strains is probably mediated through differences in production of the toxin cytochalasin B. Strains with high cytochalasin B production appear to be constrained to slower growth rates even under optimal conditions, generating the observed trade-off between pathogenicity and aggressiveness. This MGR-correlated variation in cytochalasin B production was observed even in apparently growth-limiting conditions in liquid culture, suggesting that the variation among isolates in cytochalasin B production capacity was not a proximal response to growing conditions but is instead likely to have an intrinsic genetic basis. This argument is strengthened by the evidence from solid wheat seed culture, where a similar trade-off between cytochalasin B production and MGR in solid culture was observed (Masi *et al.*, 2014a).

Cytochalasin B was not constitutively produced by *P. semeniperda* under all cultural conditions. Its production was induced only in seeds or in media that contained host seed constituents (Fig. 3a,b). This strongly suggests that it is directly implicated in pathogenesis on seeds. All 20 isolates produced large quantities of this toxin (0.3–1.4% of dry mycelial biomass), indicating that it may be an essential component of pathogenesis regardless of seed dormancy status. The fact that living dormant seeds were able to substantially suppress cytochalasin B production (Fig. 3a) indicates that toxin production

suppression could be an important component of host seed resistance. Cytochalasin B is produced by many fungi with contrasting life histories (Scherlach *et al.*, 2010). It may have multiple roles in pathogenesis in different pathosystems, and is even produced by some non-pathogenic fungi. However, in *P. semeniperda*, its role in pathogenesis on seeds seems clear, especially given the large amounts produced.

Previous experiments have shown that cytochalasin B is toxic to germinating *B. tectorum* seeds at concentrations as low as 10^{-4} M, but exogenous application of the toxin had no effect on seed germination per se (Masi *et al.*, 2014a,c). Even when seeds were injured to allow easy passage through seed coverings, no effect on germination was seen (S. Clement, unpublished data). It seems likely that the pathogen has a specific mechanism for injecting this metabolite directly into the embryo, where its cytotoxic effect can manifest itself directly within living cells.

The results of the co-inoculation experiment added the element of within-seed competition to the concept of selection for different pathogen genotypes on dormant vs. nondormant seeds. While it cannot be demonstrated that MGR was the only factor associated with differential success of the two isolates on dormant vs. nondormant seeds, the results provide provisional support for the hypothesis that MGR plays a role in the competitive outcome following mixed strain infections. On dormant seeds, the fast-germinating isolate was clearly the winner, not a surprising result, as a fast-growing strain should be able to out-compete a slow-growing strain when the two co-infect a single dormant seed (Fig. 6b). This should provide strong selection for high MGR in field seed banks. In contrast, when nondormant seeds were inoculated, the slow-growing isolate was the winner, at least on killed seeds, although the sample size was too small to confirm this result unequivocally. It is possible that a fast-growing strain could 'cheat' by taking advantage of nondormant seed resources made available through aggressive action of a slower-growing strain (e.g. Barrett *et al.*, 2011), but this effect was not seen in the present study.

Mixed strain infections appear to be common in field *B. tectorum* seed banks. When multiple stromata from individual killed seeds were genotyped (using the technique in Data S2) for 25 seeds from seed bank samples collected at the Whiterocks Exlosure in 2006, nine of the seeds (36%) bore stromata belonging to two or more SSR genotypes (S. E. Meyer, unpublished data). The maintenance of high variation in MGR despite the apparent advantage for fast-growing strains in competition on dormant seeds indicates that nondormant seed mortality in field seed banks may be more important to the success of the pathogen than would be predicted from laboratory inoculation trials (Beckstead *et al.*, 2007).

This investigation is among the first to examine the effects of temporally varying selection and resource trade-offs on traits related to fitness in a generalist soilborne plant pathogen. It demonstrates that specialization on

specific host genotypes, local adaptation and fluctuating frequency-dependent selection are not the only factors maintaining genetic variation for traits related to pathogenicity in natural plant pathogen populations, although these factors are much more commonly studied (Tack *et al.*, 2012). It also provides evidence that variation in toxin production can be tied directly to pathogen fitness outcomes, and further elucidates important differences between seed pathogens and pathogens of actively growing plants.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Data S1. Methods for extraction and quantification of cytochalasin B.

Data S2. Development and use of microsatellite markers for *Pyrenophora semeniperda*.