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Research

The quick and the deadly: growth vs virulence in a seed bank pathogen

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Summary

- We studied the relationship between virulence (ability to kill nondormant *Bromus tectorum* seeds) and mycelial growth index in the necrotrophic seed pathogen *Pyrenophora semeniperda*. Seed pathosystems involving necrotrophs differ from those commonly treated in traditional evolution-of-virulence models in that host death increases pathogen fitness by preventing germination, thereby increasing available resources. Because fast-germinating, nondormant *B. tectorum* seeds commonly escape mortality, we expected virulence to be positively correlated with mycelial growth index.
- We performed seed inoculations using conidia from 78 pathogen isolates and scored subsequent mortality. For a subset of 40 of these isolates, representing a range of virulence phenotypes, we measured mycelial growth index.
- Virulence varied over a wide range (3–43% seed mortality) and was significantly negatively correlated with mycelial growth index (R^2 = 0.632). More virulent isolates grew more slowly than less virulent isolates.
- We concluded that there is an apparent tradeoff between virulence and growth in this pathogen, probably because the production of toxins necessary for necrotrophic pathogenesis competes with metabolic processes associated with growth. Variation in both virulence and growth rate in this pathosystem may be maintained in part by seasonal variation in the relative abundance of rapidly germinating vs dormant host seeds available to the pathogen.

Introduction

The evolution of virulence has been the subject of intensive research, starting with the seminal paper of Anderson & May (1982), who proposed a model which assumed that virulence is an unavoidable negative consequence of parasite multiplication within the host. In this model, the tradeoff between increased reproductive rate and longevity of infection results in the prediction of an evolutionarily stable strategy (ESS) of optimal virulence that maximizes total pathogen reproductive output (reviewed by Alizón *et al.*, 2009). This early tradeoff model narrowly defined virulence as host death rate caused by the pathogen. Death of an animal host ends the infection for a biotroph and, if it takes place prematurely, before the pathogen has completed its life cycle or has had multiple cycles of propagule production, this clearly represents excessive virulence with a fitness cost.

Many effects of life history and population dynamics on the shape of the ESS curve have been examined using modeling approaches. These include the idea that both coinfection by genetically different strains with equal probabilities of transmission and superinfection, or the supplanting of one strain by another that is competitively superior, will lead to the evolution of increased virulence relative to that predicted for a single-strain infection (Nowak & May, 1994; May & Nowak, 1995). Another idea is that vertical transmission will lead to decreased levels of virulence, because the parasite requires the host to reproduce in order to be transmitted, but this simple result is complicated if horizontal transmission and multiple strains are present (Lipsitch *et al.*, 1996). Another model deals

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with host population structure, and leads to the prediction that virulence will decline when pathogen dispersal, and thus contact with new hosts, is reduced (Lipsitch *et al.*, 1995).

The concept of tradeoffs in virulence evolution has been broadened to include factors other than longevity as the tradeoff and host death rate as the virulence measure (Combes, 1997; Poulin & Combes, 1999). For example, O'Keefe & Antonovics (2002) modeled virulence evolution in castrating pathogens that have no impact on mortality, but where host fecundity and pathogen transmission are negatively correlated. They found that virulence could evolve to high levels in a spatially unstructured model, pushing pathogen and sometimes host populations towards extinction. The introduction of population structure allowed for stabilization at intermediate levels of virulence.

The evolution of virulence has been researched extensively for plant pathogens (reviewed by Sacristán & García-Arenál, 2008). Virulence has been defined as both pathogenicity (infectivity), which is the ability to infect specific plant genotypes, and aggressiveness, which is measured as the degree of damage to the host plant (Sacristán & García-Arenál, 2008). Here, we use the latter definition, which includes the concept that virulence can be measured in terms of negative fitness consequences for the host. These consequences often do not include outright mortality and can be difficult to quantify. Plant pathogens possess a very broad spectrum of mechanisms of pathogenesis, and it has been suggested that these mechanisms need to be considered explicitly in order to develop more refined models of virulence evolution (Frank & Schmid-Hempel, 2008).

Seed bank pathogens are a poorly studied group of organisms, in spite of their undeniable importance in plant population biology and the structuring of natural plant communities (Gilbert, 2002). These pathogens present an interesting variation on the theme of virulence vs pathogen reproductive output. Unlike an actively growing plant or animal, a host seed has finite resources, and, if the seed germinates, the resultant seedling will garner most of these resources. This should mean that a more virulent seed pathogen will have higher fitness, because rapid seed death leads to increased resources available for pathogen growth, and presumably for reproduction as well.

In this study, we investigated virulence in the generalist ascomycete seed bank pathogen *Pyrenophora semeniperda* (Medd *et al.*, 2003; anamorph *Drechslera campanulata*). This organism produces macroscopic, fingerlike, black stromata, so that diseased seeds in soil seed bank samples and inoculation trials can readily be discerned. Like its relatives, which cause economically important foliar diseases of cereal crops (e.g. *P. tritici-repentis*, *P. teres*), *P. semeniperda* is a necrotroph. These pathogens invade host tissues by secreting toxins that create a front of dead tissue which is then digested enzymatically to provide nutrition to the fungus. This mode of pathogenesis has been extensively characterized for other necrotrophs, such as *Cochliobolus* (Panaccione, 1993) and *Botrytis* (van Kan, 2006). *Pyrenophora semeniperda* also produces toxins, including cytochalasin B and other more unusual cytochalasins (Evidente *et al.*, 2002; Capio *et al.*, 2004). Toxin production in this organism is known to vary among isolates, and levels of culture filtrate toxicity in wheat seedling bioassays have been positively correlated with host leaf disease severity after needle conidial inoculation (Campbell *et al.*, 2003a). Culture filtrates in this study had no effect on intact seeds.

Pyrenophora semeniperda has been considered a weak pathogen because the cereal crop seeds that often manifest the disease in laboratory viability tests usually escape mortality and develop into normal seedlings (Campbell & Medd, 2003). We have shown, however, that the fate of a seed infected by this pathogen is largely determined by its germination rate (Beckstead *et al.*, 2007). Fast-germinating seeds, such as cereal crop seeds and nondormant seeds of weedy annual grasses, usually escape mortality and germinate normally, although the fungus is often able to sporulate on germinated seeds (Medd & Campbell, 2005). By contrast, slow-germinating or dormant seeds of susceptible species are usually killed (Kreitlow & Bleak, 1964; Beckstead *et al.*, 2010).

We have documented the major impact of this pathogen in seed banks of the invasive winter annual grass *Bromus tectorum* in semiarid North America. In the extensive monocultures of this annual grass that have replaced native vegetation over millions of hectares in the region, densities of pathogen-killed seeds as high as 20 000 m^{-2} are not uncommon (Meyer *et al.*, 2007). By contrast, the seed banks of native grasses generally contain low to very low densities of pathogen-killed seeds (Beckstead *et al.*, 2010), indicating that evolution on the host *B. tectorum* is probably the primary force shaping virulence patterns in the system studied. The impact of the disease on *B. tectorum* is most severe in spring, when remaining ungerminated seeds have entered a state of secondary dormancy and can germinate slowly if at all; nondormant seeds in autumn can germinate very rapidly and usually escape mortality (Meyer *et al.*, 2007).

In this study, we defined pathogen virulence as the ability to kill nondormant *B. tectorum* seeds. We chose this measure because dormant seeds generally suffer mortality regardless of strain identity, whereas nondormant seeds provide a more precise method of detecting virulence variation in the pathogen. Because speed is apparently of the essence in successfully killing a rapidly germinating seed, we predicted that virulence would be positively correlated with the mycelial growth rate, a prediction that is in accord with the classical virulence models described earlier. An alternative prediction was that virulence would be positively correlated

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with toxin production, but we did not test this alternative hypothesis directly.

Our first study objective was to determine the range of virulence phenotypes present in two independent sets of isolates obtained from field seed bank samples. The first set was obtained from a broad array of pathogen populations from *B. tectorum*-dominated sites in Utah, Nevada, Colorado and Idaho, USA (S. Meyer, unpublished), as well as from one population on *Bromus rubens* in northern Arizona and one population on *B. tectorum* in Turkey. The pathogen had not been reported previously to definitely occur in Eurasia, so we made an effort to obtain and screen several isolates from our Turkey collection (Stewart *et al.*, 2009). The second isolate set was later obtained from eight populations in western Utah, north-central Nevada and south-central Washington as part of a different study.

Our second research objective was to formally test the hypothesis that virulence is positively correlated with the mycelial growth rate. We used a subset of isolates selected to represent a wide range of virulence phenotypes from each of the two isolate groups to carry out this test.

Materials and Methods

Seed bank sample collection and processing

Samples were collected from *B. tectorum* L. monocultures at the sites described above for seed bank quantification in late spring using a steel can, 6 cm in diameter and 4 cm deep, which was pushed into the soil until flush with the litter surface. The can was then lifted out with a trowel and its contents were emptied into a labeled paper sack. Samples were air-dried if necessary, screened to remove excess loose soil, and hand processed to remove all seeds with protruding fungal stromata, as well as all apparently viable seeds (see Beckstead *et al.*, 2010 for more details). For the virulence trials, ungerminated *B. tectorum* (or, for one population, *B. rubens* L.) seeds with protruding *P. semeniperda* (Brittlebank and Adam) Shoemaker stromata from each pathogen population were stored air-dry at room temperature in Petri dishes until used for isolation and culturing.

Isolation and pure culturing

Virulence trials required the production of conidial inoculum from a pure culture of each strain. We used two methods to obtain pure cultures. The first method involved the removal of individual stromata from killed seeds, surface sterilization and plating onto V8 agar (Beckstead *et al.*, 2007). Most isolates produced abundant new stromata in culture, and these could be individually transferred to modified alphacel medium (MAM; Campbell *et al.*, 2003b) for conidial production. We later developed an alternative isolation method based on the observation that stromata on killed seeds will usually produce new conidia if they are incubated in water following wounding by breaking off the tip. These new conidia were then transferred to a small volume of sterile water using a needle, and the conidial suspension was poured over water agar. Excess water was decanted, and the plates were incubated for 8 h at room temperature. Single germinated conidia free of apparent contamination were then transferred using a needle under a dissecting microscope directly to MAM plates for conidial production. Most of the isolates in the first trial were obtained by the stromatal isolation method, whereas those in the second trial were obtained primarily by the single-sporing method.

Conidial production, harvest, storage and testing

For most isolates, culturing on MAM resulted in the highest conidial yields (Campbell *et al.*, 1996), although this varied by isolate in our experience, with some producing as well or better on V8 agar. We typically grew each isolate on both types of medium and pooled the resulting conidial yield. We used the protocol of Campbell *et al.* (2003b) to stimulate maximum conidial production. Isolates were incubated under white and ultraviolet light with a 12-h photoperiod, at temperatures at or near the optimum (20°C). Cultures were wounded by scraping after 5–7 d of growth. *C.* 5–7 d following wounding, the plates were harvested by rinsing with sterile water onto the surface of a 25-μm sieve. Twenty-five 5-cm plates were routinely produced for each isolate. The conidia were allowed to air-dry for several hours on the sieve, and were then scraped free and placed in a small snap-cap glass vial for storage at room temperature until initiation of the tests. Vials were left uncapped overnight to ensure that conidia had fully dried before capping. *C.* 5–10% of the isolates failed to yield conidia in culture; these isolates were necessarily excluded from further screening.

To verify viability and vigor before inclusion in virulence trials, the conidial collections were tested for germinability by preparing a conidial suspension in sterile water and casting this onto the surface of a potato dextrose agar (PDA)-coated microscope slide. Conidial germination was measured, usually after 6 h at room temperature, by counting the number of germinated conidia out of a total of 100 conidia in each of four independent passes under a compound microscope. Most collections germinated to near 100% within 6 h; isolates with very slow germination or low germination percentages (< 10% of total isolates) were excluded from the trials.

Virulence trials

Virulence trials were carried out using nondormant *B. tectorum* seeds collected at Whiterocks in Skull Valley, Utah, USA, in June 2008. Uninoculated seeds of this lot

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germinated to > 50% in 1.5 d and to 100% in < 4 d at 20°C. A total of 43 isolates was included in the first trial, whereas 35 isolates were included in the second trial. We repeated each of the two virulence trials in time, and each isolate was included as two replications in each time repeat. For each experimental unit, 3 mg of conidia were placed in a small vial with 50 seeds. These were vibrated together using a vortexer to ensure even conidial coverage on the seeds. This usually resulted in a small excess of inoculum not adhering directly to the seeds. This inoculum load is at least two orders of magnitude higher than would probably be encountered under field conditions; the reason for the high load was to attempt to detect maximum among-isolate variation in expressed virulence (seed mortality). As mentioned earlier, at inoculum levels encountered in the field, most nondormant seeds escape. The inoculated seeds for each replication were then spread on the surface of two germination blotters (Anchor Paper, St. Paul, MN, USA) saturated with water and placed in a 10-cm plastic disposable Petri dish. The dishes were stacked randomly into plastic bags to retard water loss and incubated at 20°C with a 12-h photoperiod. Dishes were watered as needed for the duration of the experiment and scored at 2, 4, 7, 11 and 14 d. At each scoring, germinated seeds and ungerminated seeds with clear disease signs (protruding black stromata) were counted and removed. At 14 d, ungerminated seeds without disease signs were scored for viability using a cut test (Oöi *et al.*, 2004); usually no ungerminated disease-free seeds remained in the dish. Virulence was calculated from these data as the proportion of total viable seeds that failed to germinate and that also developed disease signs within the 14-d incubation period. These seeds were considered to have been killed by the pathogen.

Mycelial growth rate studies

We performed a pilot growth rate study with four isolates using the protocol of Campbell *et al.* (1996) in order to determine the number of replicates and the measurement intensity necessary to obtain sufficient precision in the full-scale studies. Single germinated conidia from each isolate were inoculated into the centers of ten 10-cm Petri dishes containing quarter-strength PDA. This medium stimulates mycelial growth but not sporulation (Campbell *et al.*, 1996). It contains starches and sugars similar to those found in grass seed endosperm. The undersurface of each plate was marked with straight lines at 45° intervals across its diameter, providing four axes of measurement for colony diameter. Colony margins were clearly visible from the underside of the plate. We confirmed that, at constant temperature, mycelial colony diameter increased linearly with time, and determined that the slope of increase in colony diameter was almost perfectly correlated with day-14 colony diameter (df = 2, $r = 0.999$, $P < 0.01$). We therefore decided to use day-14 colony diameter as our index of mycelial growth rate (MGI). We also determined that five replications per isolate provided sufficient precision for distinguishing differences in growth rate among isolates. Full-scale trials were performed using the protocol of five plates per isolate and four colony diameter measurements along the premarked axes after 14 d of incubation at room temperature (*c.* 22°C).

We used data from each virulence trial to select a subset of isolates representing the full range of virulence phenotypes for mycelial growth rate studies. Eighteen isolates from the first virulence trial were included in the first round of growth rate studies. In the second round, we included 20 new isolates. As a check between the two growth rate studies, we also included two isolates that had been included in the first round, for a total of 22 isolates.

Statistical analysis

For each virulence trial, we carried out mixed-model ANOVA with isolate as the fixed effect and time repeat as the random effect. We also performed mixed-model ANOVA on the combined dataset to test for a difference in virulence between the two isolate groups. Proportional virulence data were arcsine square root transformed before analysis to improve variance homogeneity. We also prepared frequency histograms showing the distribution of isolates into nine virulence categories for each trial.

We analyzed the relationship between MGI and mean virulence using analysis of covariance, with MGI as the dependent variable, mean virulence (seed mortality percentage) as the continuous independent variable and growth rate trial as the class variable. MGI was chosen as the dependent variable because it appeared to vary as a function of trial, allowing variance caused by the effect of trial to be included in the model. We had no a priori reason to assign causality to either variable.

Results

Virulence trials

Virulence varied significantly among isolates in both virulence trials, with a range from 3.0% to 43.3% mean seed mortality in the first trial and from 9.5% to 38.1% in the second trial (Trial 1: df = 42, 85, $F = 6.24$, $P < 0.0001$; Trial 2: df = 34, 66, $F = 3.40$, $P < 0.0001$). Seed mortality did not vary significantly between virulence trials, averaging 19.4% in the first trial and 21.2% in the second trial (trial main effect: df = 1, 259, $F = 1.45$, $P = 0.2294$). We observed no significant difference between time repeats and no significant interaction between isolate and time repeat in the first trial, but, in the second virulence trial, both of these effects were highly significant, with the mean virulence

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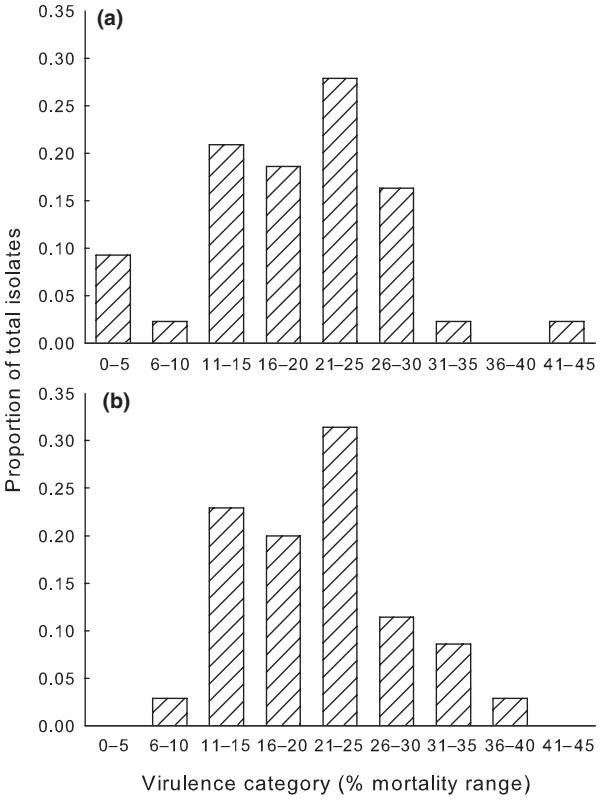

Fig. 1 Frequency distribution among nine virulence categories for *Pyrenophora semeniperda* isolates included in each of two virulence screening trials on nondormant *Bromus tectorum* seeds: (a) virulence trial 1 (n = 43); (b) virulence trial 2 (n = 35).

increasing from 18.2% to 24.6% between time repeats (time repeat main effect: df = 1, 66, F = 18.85, P < 0.0001). Eleven isolates showed increases of at least 10% in the second time repeat, but four isolates showed decreases of at least this magnitude (time repeat by isolate interaction; df = 31, 66, F = 4.21, P < 0.0001). The reason for these differences is not known, but they indicate that virulence expression was very sensitive to some uncontrolled environmental variable in the second trial, and that this sensitivity varied by isolate.

The frequency distribution of isolates in different virulence categories was quite similar between trials (Fig. 1a,b). In the first trial (n = 43), the frequency distribution was slightly right skewed, indicating a slight preponderance of less virulent isolates. In the second trial (n = 35), the distribution was closer to a normal distribution. In both trials, the great majority of isolates showed low to intermediate virulence, with only six of a total of 78 isolates, or 7.7%, exhibiting virulence > 30%.

Mycelial growth rate studies

MGI was significantly negatively correlated with virulence percentage in both growth rate trials, indicating that isolates exhibiting lower virulence were faster growing, whereas those that exhibited higher virulence grew more slowly (Fig. 2; ANCOVA virulence percentage main effect: df = 1, 37, F = 39.84, P < 0.0001). The slope of the relationship between virulence percentage and MGI (−0.4354 mm per virulence percentage point) was not significantly different between trials (ANCOVA virulence percentage × growth rate trial interaction: df = 1, 37, F = 0.33, P = 0.5702). ANCOVA accounted for 63.2% of the total variance.

There was a difference in mean MGI between growth rate trials that approached statistical significance (df = 1, 37, F = 4.04, P = 0.0518). This mean MGI difference (mean difference in day-14 colony diameter) between trials was 7.85 mm; it is apparent in Fig. 2 as a difference in the elevation of the plotted regression lines. Isolates in the second trial grew more quickly, on average, than those in the first trial. The most likely explanation for this is a difference in mean temperature between the two trials, which took place at laboratory temperature and not in a controlled environment chamber. This interpretation is supported by the fact that two isolates common to both growth rate trials both showed increased growth rates in the second trial (Fig. 2). One isolate in the second growth rate trial was anomalous in having a very slow growth rate but also relatively low virulence. This outlier is plotted in Fig. 2 but not included in the analysis. No isolates exhibited both a fast growth rate and high virulence.

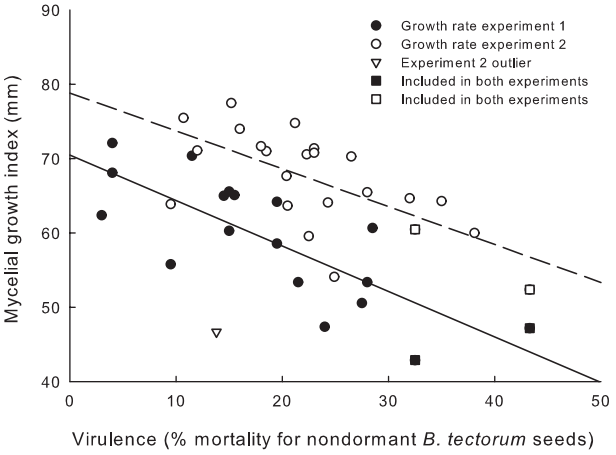

Fig. 2 The relationship between virulence (percentage of nondormant *Bromus tectorum* host seeds killed) and MGI (mycelial growth index, defined as day-14 colony diameter) for isolates in two independent growth rate trials (open vs closed symbols) conducted at laboratory temperature. Plotted lines are from regression analysis by trial. Analysis of covariance showed that the effect of virulence on MGI (overall regression slope) was highly significant, the slopes of the regression lines for the two trials were not significantly different and the elevations (y-intercepts) for the two trials were marginally significantly different. See text for statistical analysis.

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Discussion

Our hypothesis that MGI and virulence would be positively correlated, as predicted by classical virulence evolution models, was not supported. Instead, we found a highly significant negative relationship between these two variables in each of two independent experiments. This suggests that there is likely to be an underlying physiological basis for this negative relationship that is not just a function of the growing conditions or the particular isolates selected. Our best hypothesis is that variation among isolates in the ability to kill rapidly germinating host seeds is related to variation in the production of the toxins necessary for pathogenesis. Virulence and toxin production have been found to be positively correlated in several necrotrophic pathogens, including *Botrytis cinerea* (Reino *et al.*, 2004), *Bipolaris sorokiniana* (Apoga *et al.*, 2002) and *Gibberella fujikuroi* (Desjardins *et al.*, 1995), and, as mentioned earlier, virulence in leaf inoculations with *P. semeniperda* has been positively correlated with culture filtrate toxicity as measured by wheat seedling bioassay (Campbell *et al.*, 2003a). If toxin biosynthesis requires a high investment of resources, it could compete with metabolic processes associated with growth, resulting in the tradeoff between virulence and growth rate observed. It may be that the injection of large amounts of toxins, or possibly of more resource-expensive toxins, is needed to kill nondormant host seeds, and that strains that can cause this mortality are inherently handicapped in terms of growth rate.

Although a negative relationship between virulence and growth rate is not necessarily unexpected for a necrotrophic pathogen, we were unable to find any published confirmation of this phenomenon. In one of the few studies examining this question, *in vitro* growth rate of the pathogen *Alternaria brassicicola* was weakly positively correlated with aggressiveness (lesion expansion rate on the host *Cakile maritima*) on an individual isolate basis, and quite strongly positively correlated with the rate of epidemic development at the population level (Thrall *et al.*, 2005). Interestingly, these workers also found a positive correlation between mycelial growth rate and total spore production *in vitro*, but a negative correlation with spore production per unit of colony area. They interpreted this as a possible tradeoff between growth and reproductive output, showing that these two measures of fitness may not always be positively correlated, and providing a possible selective force for the maintenance of growth rate variation. We have not yet examined the relationship between growth rate and reproductive output in *P. semeniperda*. The elucidation of the relationship of toxin production to virulence, growth rate and reproductive output will be the essential next steps in understanding the evolution of virulence in this pathogen.

For the purposes of distinguishing among virulence and growth rate phenotypes in our study, we made several decisions that limit the ability to generalize from our results. First, by eliminating isolates that failed to sporulate or that produced low-vigor conidia from our trials, we may have introduced bias, although isolates eliminated for these reasons represented a small fraction of the total. Second, we measured pathogen growth rate *in vitro* rather than *in vivo*, and we did not quantify reproductive output independently from growth. Third, we used high inoculum loads. We do not yet know the implications of virulence variation at the lower inoculum levels more typically found in *B. tectorum* field seed banks (Beckstead *et al.*, 2010). Fourth, we screened for virulence on fast-germinating, nondormant seeds, which may not be the primary target of attack in the field.

There is apparently an optimal level of virulence in *P. semeniperda* that is far below the maximum level observed, based on the frequency distributions of virulence phenotypes in Fig. 1. However, the underlying selection pressures constraining virulence levels could be quite different from those proposed in classical models. There should be a clear fitness advantage to higher virulence when the prey is a rapidly germinating seed that must be quickly disabled to maximize resource availability to the pathogen. However, if the seed under attack is dormant, there is no apparent advantage to either high virulence or a fast growth rate, because the pathogen is likely to win the resource race regardless of which trait it possesses. Changes through the season each year in the relative frequency of different host seed dormancy phenotypes may be a primary selective force maintaining the virulence polymorphism observed (Pfennig, 2001).

The tradeoff between virulence and growth rate may become more important when multiple strains of the pathogen infect a single seed. We know that this is a common occurrence from molecular genetic work in which cultures from multiple stromata on single seeds from field seed banks were characterized using microsatellite or single sequence repeat markers (S. Meyer, unpublished). If two strains infect the same dormant seed, the strain with the fastest growth rate would be expected to have a fitness advantage, as it could usurp resources from a more virulent, but slower growing, coinfecting strain (de Nooij & van Damme, 1988). This would tend to select for lower virulence. The situation becomes even more complex when two contrasting strains coinfect a rapidly germinating seed. A fast-growing strain with low virulence could reap most of the rewards of seed mortality caused by a more virulent but slower growing coinfecting strain, rewards that would not have been made available without mortality caused by the more virulent strain. The determination of fitness outcomes for contrasting coinfecting strains will require quantitative molecular genetic

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examination of stromata from experimentally coinfected seeds.

Our study suggests that the upper and lower limits of virulence in this pathosystem are probably set by physiological constraints. A minimum level of toxin production is probably necessary to carry out the process of necrotrophic pathogenesis, even on dormant seeds, so that all strains will possess at least this minimum level. Moreover, there is probably an upper limit to the level of resources that can be invested in toxin production and still permit the organism to carry on the metabolic processes necessary for growth and reproduction. However, exactly where the balance is struck between virulence and growth rate appears to depend on a complex interplay between the infecting strain, the status of its host seed and the attributes of coinfecting strains. Whether there is truly an ESS for optimal virulence in this pathosystem remains to be determined through more detailed experimental work. It seems apparent, however, that, like the castrating pathogens discussed by O'Keefe & Antonovics (2002), in terms of virulence evolution, necrotrophic seed bank pathogens could be playing by a different set of rules.

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